

Toxoplasma gondii Detection in Domestic and Wild Animals: Methods
Development and Application for Epidemiological Investigations

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

John Schaefer

May 2013

© 2013 John Schaefer

Toxoplasma gondii Detection in Domestic and Wild Animals: Methods Development
and Application for Epidemiological Investigations

John Schaefer, Ph.D.

Cornell University 2013

Toxoplasma gondii infection remains a condition of interest in both human and veterinary medicine despite having recently celebrated its first century of recognition. *Toxoplasma* is distinguished from intestinal coccidia in its utilization of intermediate hosts, causing disease in a wide spectrum of animals. Diagnosis is also more complicated as it localizes in numerous tissues in intermediate hosts and has only a brief period of oocyst shedding in infected felids. The inability to diagnose *Toxoplasma* infection by direct observation has led to a reliance on serological assays as the primary diagnostic method. Serological testing establishes a history of exposure to *Toxoplasma*, but does not definitively identify this as the genesis of the clinical signs of interest. The situation is more complicated in veterinary medicine by the decreasing availability of test methods that can be used across the range of animals susceptible to infection.

The development of protein conjugates A, G and chimeric A/G allows the use of a human enzyme-linked immunosorbent assay for a range of animal species. When compared to established agglutination testing for the detection of anti-*Toxoplasma* IgG antibody, agreement between the two tests was determined to be very good, with a kappa value of 0.83 for the trial using proteins A and G separately and 0.81 when

chimeric protein A/G was used. This technique demonstrated a seropositivity of 38.5% when utilized to examine the occurrence of *Toxoplasma* exposure among hunter-killed white-tailed deer in New York State from 2010, indicating a notable level of exposure in this population. Further analysis revealed older deer are more likely to have seroconverted, consistent with a model of significant horizontal transmission by oocysts. No significant differences in infection based on sex or local human population density were noted. A quantitative real-time PCR assay was developed based on the *Toxoplasma B1* gene, but did not detect the presence of parasite DNA in serum samples from seropositive dogs and sheep. These ELISA and PCR assays are valuable both to veterinary diagnosticians in individual cases and to investigators studying *Toxoplasma* prevalence in a broad spectrum of domestic and wild animals.

BIOGRAPHICAL SKETCH

John Schaefer received his Bachelor of Arts degree in Geology from Haverford College. He received his Doctor of Veterinary Medicine from Texas A&M University and entered private practice in the Houston area, gaining extensive and often unwanted experience in clinical parasitology. He received a Master of Science degree in Entomology from the Ohio State University, with an emphasis in veterinary acarology and tick-borne diseases. He began investigating toxoplasmosis as a research associate and then Ph.D. student in the Parasitology section of the New York State Animal Health Diagnostic Center, which, along with Cornell University's Veterinary College and the United States Department of Agriculture provided generous support to allow the completion of the described work.

ACKNOWLEDGMENTS

Dr Susan Wade, my advisor and committee chair, provided invaluable direction and support throughout my doctoral education and research. Dr Hussni Mohammed has been an integral part of our research team and I appreciate his frank advice as a collaborator and committee member. Dr Craig Altier generously provided the use of his laboratory and the assistance of his former Ph.D. student Dr Chien-che Hung in this research. Dr Judith Appleton has given beneficial advice in guiding the later stages of this work. Dr Eric Denkers provided cultured tachyzoites from his laboratory to allow molecular investigation. Dr Sharon Patton and Aly Chapman instructed me in agglutination techniques. Drs. Elizabeth Bunting, Megan Kirchgessner and Christopher Whipps provided samples and direction for the white-tailed deer project. Dr Bruce Akey and the New York State Animal Health Diagnostic Center provided generous support for the project. Current and former members of the Parasitology Section provided excellent assistance: Danielle McGuire, Stephanie Schaaf, Pixie Senesac, Katrina Walker and Holly White.

I have had the good fortune to have had encouraging mentors throughout my academic training, notably Dr Bill Stich, Dr Glen Needham, Dr Hans Klompen and Dr Yasuko Rikihisa from the Ohio State University. My family has been a great support during this project, notably Dr Deanna, Joanna and Lia Schaefer.

This work was supported by the USDA federal formula fund grant NYC-478430, and a Graduate Research Assistantship from Cornell University's College of Veterinary Medicine.

TABLE OF CONTENTS

BIOGRAPHICAL SKETCH.....	iii
ACKNOWLEDGMENTS.....	iv
LIST OF FIGURES.....	vi
LIST OF TABLES.....	viii
CHAPTER ONE	
Introduction.....	1
CHAPTER TWO	
Modification of a commercial <i>Toxoplasma gondii</i> immunoblobulin G enzyme-linked immunosorbent assay for use in multiple animal species.....	24
CHAPTER THREE	
Chimeric protein A/G conjugate for detection of anti- <i>Toxoplasma gondii</i> immunoblobulin G in multiple animal species.....	40
CHAPTER FOUR	
<i>Toxoplasma gondii</i> seroprevalence in New York State white-tailed deer (<i>Odocoileus virginianus</i>).....	52
CHAPTER FIVE	
PCR development and detection of <i>Toxoplasma gondii</i> DNA in infected dogs (<i>Canis familiaris</i>) and sheep (<i>Ovis aries</i>).....	66
CHAPTER SIX	
Conclusion.....	83

LIST OF FIGURES

FIGURE 2.1

Effect of enzyme-linked immunosorbent assay optical density (OD) ratio cut-off value on relative sensitivity and specificity.....33

FIGURE 2.2

Receiver operating characteristic calculation for modified immunoglobulin G enzyme-linked immunosorbent assay.....33

FIGURE 2.3

Receiver operating characteristic calculation by species.....34

FIGURE 3.1

Variation in sensitivity and specificity relative to enzyme-linked immunosorbent assay optical density(OD) ratios.....47

FIGURE 3.2

Receiver operating characteristic calculation for modified immunoglobulin G enzyme-linked immunosorbent assay.....47

FIGURE 4.1

Map of *Toxoplasma* seropositive and seronegative white-tailed deer and local human population density.....60

FIGURE 5.1

Results of traditional PCR testing of positive control using primers for 529 repeat element, B1 gene, and newly developed primers 529js1 and 529js2.....74

FIGURE 5.2

B1 gene and 529js1 evaluation of extracted canine and caprine sera.....74

FIGURE 5.3

Lack of inhibition of negative serum (canine).....74

FIGURE 5.4

Amplification plot with particles per reaction noted.....77

FIGURE 5.5

Standard curve for dilution series.....77

FIGURE 5.6

Melt curve.....78

LIST OF TABLES

TABLE 2.1

Comparison of indirect hemagglutination assay to modified immunoglobulin G enzyme-linked immunosorbent assay testing.....35

TABLE 2.2

Kappa value calculation by species.....35

TABLE 3.1

Comparison of indirect hemagglutination to modified IgG ELISA.....48

TABLE 3.2

Results of modified IgG ELISA testing by species.....48

TABLE 3.3

Comparison of repeated assays using modified IgG ELISA.....48

TABLE 5.1

DNA quantitation in extracted Toxoplasma culture and serum samples.....72

TABLE 5.2

Primers used to amplify indicated target.....72

CHAPTER ONE

INTRODUCTION

Toxoplasmosis is a significant disease in humans and many other animal species and is caused by the invasion of tissue by the apicomplexan *Toxoplasma gondii*. While the systematic description of the organism is rather straightforward, namely being the only representative of the genus *Toxoplasma* and the small family Toxoplasmatidae in the order Eimeriorina, there are not many other facets of *Toxoplasma* and toxoplasmosis that are so easily characterized (Dubey, 2010). This has contributed to both an intense research and public interest in the organism and its associated disease, and the generation of misunderstanding and misplaced concern in the medical and veterinary communities. A singular aspect of the parasite's life cycle that is well understood is the importance of domestic and wild felids in the perpetuation of *Toxoplasma* as the only known definitive hosts. Beyond that, the ability of the organism to be transmitted both vertically and horizontally, infecting new hosts through ingestion of tissue cysts in intermediate hosts, oocysts in contaminated soil, water and produce, and from congenital transmission, makes a simple portrayal of the typical *Toxoplasma* life cycle a fleeting generality. Much of the interest in toxoplasmosis relates to the severity of disease in human fetuses and immunosuppressed populations (Lindsay *et al.*, 2003). Though only an estimated 15% of human infections with *Toxoplasma* result in detectable clinical signs, it is the third leading cause of death from foodborne disease in the United States (Mead *et al.*, 1999), hinting at the prevalence of infection in this population. Understanding the behavior of *Toxoplasma* in human populations gives a woefully incomplete picture of the parasite. Since it is a zoonotic disease, medical and

veterinary investigations should be intimately linked to produce reasonable control strategies. This study describes the creation and testing of new diagnostic strategies to assist veterinary investigators in their understanding of the role of *Toxoplasma* infection in their patients, and thereby contributes to efforts to combat its further perpetuation.

HISTORY

The history of *Toxoplasma gondii* discovery, identification of hosts, description of its life cycle, diagnostic strategies and treatment have been thoroughly reviewed (Dubey 2008; Dubey 2010; Ferguson, 2009), however a summary is warranted to distill the pertinent literature that influenced the current investigation. Two investigative teams in the early 20th century noted the presence of protozoal organisms in their research gundy (*Ctenodactylus gundi*) in Tunisia and rabbits (*Oryctolagus cuniculus*) in Brazil, and both initially attributed the observation to *Leishmania* sp., before the former group realized it was an undescribed species and the former group named it *Toxoplasma gondii* (*sic?*) (Nicolle and Manceaux, 1909; Splendore, 1908). One year later, a clinical case of toxoplasmosis was described in the domestic dog (*Canis familiaris*) in Italy, an early insight into the distribution of this parasite across divergent animal species and geographic areas (Mello, 1910). Tissue cyst structures were first noted by a group in rabbits and mice and described cytologically about 20 years after the organism's initial identification (Levaditi *et al.*, 1928). Early investigators depended on thorough microscopic work and staining techniques. The advent of serological tests and molecular diagnostic techniques led to a rapidly increased understanding of the parasite and its role in clinical disease.

Human Toxoplasmosis

Toxoplasmosis was established as a disease of humans by the observation of a case of congenital transmission in 1939 and infectiousness to animals from this case was demonstrated (Ferguson, 2009; Wolf *et al.*, 1939). The recognition of the potential importance of *Toxoplasma* infection in human health precipitated development of diagnostic tests to allow antemortem detection. Sabin and Feldman recognized that *Toxoplasma* tachyzoites lose their affinity for methylene blue in the presence of anti-*Toxoplasma* antibody, leading to the description of the first widely used serological test, the Sabin-Feldman dye test (Dubey, 2008; Sabin and Feldman, 1948). Though still considered a gold standard in serological testing, the requirement for live parasites limits the utility of the test, both due to the difficulty of acquiring and storing the organism, and potential risk to laboratory personnel. Multiple tests have since been developed that observe the agglutination of killed parasite or particles coated with antigen in the presence of antibody as the basis of serological diagnosis, including the direct agglutination test, the modified agglutination test, indirect hemagglutination and latex agglutination (Desmonts and Remington, 1980; Dubey and Desmonts, 1987). These have the advantages of not using live organism, but still being able to test human and animal sera equally well, due to lack of reliance on a species-specific protein conjugate for detection. A disadvantage is their reliance on subjective interpretation by the user as to a positive or negative reaction. The reliance on serological interpretation in the diagnosis of toxoplasmosis leads to difficulties for medical and veterinary clinicians in judging the significance of a positive test, *i.e.*, is the presence of antibody indicative of a clinically significant infection or just a historical artifact of an old infection?

The advent of techniques to establish the presence of live organism in different tissues holds promise in providing more information, with a polymerase chain reaction technique targeting *Toxoplasma* first being described over two decades ago (Burg *et al.*, 1989).

Feline Toxoplasmosis

Feline toxoplasmosis was first diagnosed in a domestic cat (*Felis catus*) from Middletown, NY, in 1942 (Dubey 2008; Olafson and Monlux, 1942). The protracted time between initial description (1908) and this first described case in what was later determined to be the most significant definitive host of the organism is a tribute to the covert nature of *Toxoplasma* transmission and symptomatology in this host. It was another two decades before an investigator noted that *Toxoplasma* oocysts can be isolated from feline feces (Hutchison, 1965). He further noted that they can remain infectious in the environment for at least 12 months as demonstrated by experimental transmission to mice. Feline intestinal stages, both sexual and asexual, were described in detail by Frenkel and Dubey, (Frenkel *et al.*, 1970). They demonstrated that domestic cats shed oocysts following ingestion of infected mice, and further noted a discrepancy in the pre-patent period for *Toxoplasma* in the feline host based on the stage of the parasite that produced the infection. Tissue cyst ingestion, which is thought to be the most common method of feline infection due to predation of intermediate hosts, revealed shedding of oocysts in 3-5 days. In contrast, ingestion of tachyzoites produced oocyst shedding in 8-10 days and ingestion of oocysts produced shedding in 21-24 days. Though oocyst shedding is intensive, with one study noting up to 13 million oocysts per gram of feces, it is temporally abbreviated, at approximately one week

(Dubey 2010; Schares *et al.*, 2008). This unfortunately makes direct fecal examination a diagnostic test of very low sensitivity even in the definitive host, and questionable specificity due to the similar appearance of other protozoan oocysts like *Hammondia hammondi*.

***Toxoplasma* in Intermediate Hosts**

The search for intermediate hosts for *Toxoplasma gondii* has been accomplished by numerous investigators with notable success (Dubey, 2010). Current evidence indicates that any warm-blooded animal can serve as an intermediate host for the organism, and the identification of marine invertebrates as a source of infection significantly broadens the number of animals of potential interest (Lindsay *et al.*, 2004). The earliest animals identified as intermediate hosts of *Toxoplasma gondii* were the guinea pig, rabbit, domestic dog and mouse (Levaditi *et al.*, 1928; Mello 1910; Nicolle and Manceaux, 1909; Splendore, 1908). The first isolation of viable *Toxoplasma* was from laboratory mice and led to its characterization as an obligate intracellular parasite (Sabin and Olitsky, 1937). The lack of a requirement for a definitive host in the parasite's propagation was noted by oral infection of pigs with mouse tissue, and of mice with pork, also establishing the potential risk to humans from meat consumption (Weinman and Chandler, 1954). Similarly, minced fetal cotyledons from suspected ovine abortion cases were demonstrated to be infectious to other pregnant ewes and mice by intraperitoneal injection (Hartley and Marshall, 1957). More recently, the surprising finding of toxoplasmosis being a cause of significant mortality in southern sea otters (*Enhydra lutris nereis*) demonstrated how pervasive environmental contamination with *Toxoplasma* oocysts is in both terrestrial and marine environments (Cole *et al.*,

2000). The ability of *Toxoplasma* to infect a wide host range and to have a broad geographic distribution (described from all continents) makes it one of the most common protozoal parasites in the world.

LIFE CYCLE

The demonstration of successful *Toxoplasma* transmission by congenital, carnivorous and fecal-oral routes stimulated research interest into the parasite's life strategy that allows survival in widely diverse hosts (Dubey *et al.*, 2008; Frenkel *et al.*, 1970; Hutchison, 1965; Weinman and Chandler, 1954). In intermediate hosts, sporozoites released from sporocysts penetrate through intestinal epithelial cells and migrate to the lamina propria, where numerous different types of cells are invaded, and two tachyzoites are produced by approximately 12 hours post infection. Following invasion of additional tissues with tachyzoites, tissue cysts have been noted to start forming by 6 days post infection and these likely persist for the life of the animal. The life cycle in felids can follow this same course, making felids uniquely both potential intermediate and definitive hosts. The latter due to their hosting the sexual stages of *Toxoplasma*, in which schizogony is noted after intestinal invasion, leading to the production of sexual stages (female macrogamonts and male microgamonts) by 2 days after infection. Following the formation of zygotes and oocyst walls, the host cell ruptures, discharging oocysts into the intestine (Dubey, 2010). Both life cycles require an evasion of the host's immune system to proceed successfully, and the same white blood cells that respond to the presence of the parasite are utilized to spread infection

throughout the host (Denkers *et al.*, 2012). The prepatent period between oral ingestion and shedding of oocysts in domestic cats varies depending on the stage of the parasite initiating infection as noted above (Frenkel *et al.*, 1970). Considering the abbreviated prepatent period following tissue cyst ingestion in the cat with the similarly brief time from oral ingestion to tissue cyst production in intermediate hosts, it is easy to surmise how quickly contamination can spread through a felid-intermediate host/prey ecosystem, e.g. felids and rodents.

PUBLIC HEALTH

Mortality due to *Toxoplasma* infection in humans occurs in very few cases, most notably due to reactive encephalitis in immunocompromised patients and abortion in naïve mothers on first exposure (Dabritz and Conrad, 2010). However, morbidity due to toxoplasmosis can take many forms, related to the diverse tissue types that can be invaded in intermediate hosts. The prevalence of infection in humans varies widely, with a seroprevalence of 38% noted in the United States and a range of 4-92% noted in other countries. The fecundity of oocyst production (an average of 10,000,000 oocysts shed per infected cat has been estimated) and their environmental durability, with survival times of greater than one year noted in both natural and experimental setting, contribute to the common nature of the infection (Dubey, 2010). However, in a large European study, contact with domestic cats was not identified as a risk factor for seropositivity. Rather, eating undercooked meat, contact with soil and travel outside of Europe, the United States and Canada were significant. In all, 30-63% of infections

were attributed to consumption of undercooked or cured meat and 6-17% of infections related to soil contact (Cook *et al.*, 2000). It should be noted that this is not necessarily conclusive, and a significant prevalence in people who don't consume meat indicates that not only oocyst contact through soil, but consumption of contaminated water, fruits and vegetables can be risk factors. Methods to reduce human exposure include: proper cooking of meat and vegetables, hand hygiene after handling meat or having soil contact, preventing predation by owned cats, providing litter boxes and controlling feral cat populations (Dabritz and Conrad, 2010). Unfortunately, even given promotion of these control measures in human and domestic animal populations, the recognition of *Toxoplasma* strains in wildlife capable of infecting humans implies a sylvatic life cycle that can independently perpetuate the parasite's population (Dubey *et al.*, 2008; Wendte *et al.*, 2011).

ANIMAL HEALTH

The occurrence of *Toxoplasma* infection in domestic cats is a topic of interest primarily due to the threat their infection poses to humans and other animals, as clinical signs in the felid host are typically inapparent. An extensive survey of cats that were clinically ill from any cause in the United States found a seroprevalence of 31.6% overall, with the southwestern US having the lowest prevalence (16.1%) and the northeastern US having the highest prevalence (43.5%). Significant factors other than geographic location that predisposed the cats to being positive were: older age, being male and being a domestic short-haired breed (Vollaire *et al.*, 2005). A study of Ohio

cats brought into a mobile spay and neuter clinic revealed a 48% overall seropositivity, with a 62% prevalence noted in owner described outdoor cats (Dubey *et al.*, 2002). A survey of Pennsylvania cats from a periurban environment yielded a significantly lower seropositivity of 19.5% (Dubey *et al.*, 2009). In the United States, the range of seropositivity survey results for cats was a low of 12.1% from Florida and a high of 80% from Iowa (Hill *et al.*, 1998; Luria *et al.*, 2004). Surveys from other countries produced a wider range of seroprevalences, from a low of 2.1% in a Chinese study to a high of 89.3% in a Colombian study (Dubey 2010; Shen *et al.*, 1990). A survey of non-domestic felines in the United States found a prevalence of 43.6%, looking at both captive and free-ranging animals (Spencer *et al.*, 2003). The degree of exposure to *Toxoplasma* for both domestic and wild felids as demonstrated by these studies is significant from diverse geographic and demographic areas.

It is noted that the description of *Toxoplasma* infection in the domestic dog preceded that in the domestic cat by more than three decades (Mello, 1910; Olafson and Monlux, 1942). While the evaluation of the significance of toxoplasmosis in the dog is somewhat complicated by the potential of infection with *Neospora caninum*, it is clear that both can be significant diseases in the dog. An examination of stray dogs and cats in Sao Paulo, Brazil, revealed a seroprevalence of 40% in cats and 50.5% in dogs, which the authors attributed to the more selective palate of cats (Meireles *et al.*, 2004). A demonstration of the broad appetite of dogs was the identification of viable *Toxoplasma* oocysts in canine feces in a German study, which could have wrongly indicted dogs as an alternative definitive host if the investigators weren't familiar with their coprophagic habits (Schaes *et al.*, 2005). A study of stray animals in Bangkok,

Thailand, revealed significantly lower levels of seroconversion than many studies, 11% in cats and 9.4% in dogs, the authors noted that this population of animals were often fed cooked fish and rice by local monks, foods that would be expected to carry a low risk of serving as routes of infection (Jittapalapong *et al.*, 2007). A study in the United States revealed a seroprevalence of 25% in Kansas dogs, with a range of 5.2-88.5% noted in other areas of the world, the lowest value noted from urban, owned dogs in Brazil and the highest value from dogs in Mato Grosso, the Brazilian state with the lowest human population density (deSouza *et al.*, 2003; Lindsay *et al.*, 1990; Santos *et al.*, 2009; Wikipedia, 2013). Domestic dogs, especially free living dogs that are more likely to participate in predation, can serve as a good sentinel for local *Toxoplasma* population, as they can be expected to be exposed both through oocyst and tissue cyst ingestion (Meireles *et al.*, 2004).

There is interest in *Toxoplasma* infection in food animals, both due to potential public health risk from meat and milk consumption and economic losses due to disease. One of the earliest demonstrations of the importance of toxoplasmosis in sheep production was an investigation of an abortion storm on a farm in New Zealand, in which minced cotyledons from infected ewes transmitted *Toxoplasma* to both additional ewes and mice (Hartley and Marshall, 1957). A similar outbreak in New York revealed that 73.8% of sheep on the farm were seropositive for *Toxoplasma* exposure (Dubey and Welcome, 1988). The range of seroprevalences in studies of sheep in the United States is 27.1-73.8%, with a range of 3-95.7% noted from sheep studies worldwide (Dubey 2010; Dubey *et al.*, 2008; Zaki, 1995). Similar clinical effects have been noted in caprine toxoplasmosis, with US surveys noting a seroprevalence of 22.1-51.9% in

goatherds, and a worldwide seroprevalence of 3.2-90.9% (Dubey 2010; Dubey and Adams, 1990; Garcia-Vazques *et al.*, 1993; McSporran *et al.*, 1985; Patton *et al.*, 1990). Consumption of poorly cooked lamb and goat should be considered a significant route to *Toxoplasma* exposure. Pork may also be a significant source of *Toxoplasma*, as reported in a Massachusetts study in which 51 of 55 pigs destined for human consumption were found to have viable tissue cysts in their heart and tongue tissue (Dubey *et al.*, 2002).

Toxoplasmosis is not considered a pathogen of significant concern in cattle and horses, though both can experience infection. These infections tend to be insignificant in both intensity and duration with misdiagnosis due to infections with *Neospora spp.* and *Sarcocystis spp.* (Dubey, 2010; Dubey and Desmonts, 1987). Many wildlife species, however, are both susceptible to *Toxoplasma* infection and clinical toxoplasmosis. One species of interest as a sentinel host for domestic herbivores is the white-tailed deer (*Odocoileus virginianus*), both due to its tendency to graze alongside livestock and its wide dispersal. A study in Ohio noted a seroprevalence of 44% among white-tailed deer, 6% in deer harvested in urban parks (Columbus) and 55% in more rural Hocking County. This implies that human (and by extension domestic cat) population density is not the only predictor of environmental contamination by *Toxoplasma* (Crist *et al.*, 1999). This question is revisited in the serosurvey of New York deer described below. A higher seroprevalence (60%) was noted in hunter-killed deer in Pennsylvania, with surveys in other states ranging from 30-64.2% (Dubey, 2010; Dubey *et al.*, 2009; Humphreys *et al.*, 1995; Vanek *et al.*, 1996). The clinical effect of

Toxoplasma infection on deer is poorly understood, as is the question of bias arising from an interaction between infection and the tendency to be killed by hunters.

DIAGNOSTIC TEST DEVELOPMENT

The discovery and subsequent investigation of *Toxoplasma gondii* largely depended on bioassays and microscopic examination until the advent of the Sabin-Feldman dye test, which allowed highly accurate antemortem diagnosis of *Toxoplasma* exposure through the evaluation of serum samples (Sabin and Feldman, 1948). The dye test proved highly sensitive and accurate, but had the distinct disadvantage of having the requirement for live *Toxoplasma* tachyzoites, a potential risk to laboratory staff conducting the test (Reiter-Owona *et al.*, 1999). In medical diagnostics, a number of immunofluorescence and enzyme-linked immunosorbent assays (ELISA) were developed but have limited utility in veterinary diagnostics due to their requirement for species-specific protein conjugate. The development of the direct agglutination and later the modified agglutination tests, techniques that utilized formalin-killed rather than live tachyzoites and did not have a conjugate requirement was revolutionary in the diagnosis and investigation of toxoplasmosis in animals (Desmonts and Remington, 1980; Dubey and Desmonts, 1987). These techniques have been further modified to produce additional agglutination tests that utilize the same strategy: the indirect hemagglutination test using antigen coated sheep red blood cells and the latex agglutination test using antigen coated latex beads. Indirect hemagglutination test results were noted to agree with Sabin-Feldman dye test results in human patients, and

similarly remain positive for life (Lunde, 1973). In a study of experimental infection using cats that were monitored for up to 950 days after inoculation, the Sabin-Feldman dye test, modified agglutination test (MAT), indirect hemagglutination test (IHA) and latex agglutination test (LAT) were compared and the MAT was found to be the most sensitive of the agglutination methods (Dubey and Thulliez, 1989). The MAT and IHA were found to produce similar results in a study of dairy goats (Patton *et al.*, 1990). The MAT also had good correlation with an ELISA test developed specifically for porcine testing (Gamble *et al.*, 2005). None of the agglutination tests reliably detect IgM on known IgM positive samples, and therefore become positive later in the course of infection than the dye test (Lappin and Powell, 1991). Agglutination testing remains the most commonly used modality for the diagnosis of toxoplasmosis in animals, despite the loss of availability of some commercial kits.

Diagnosis of *Toxoplasma* infection in humans is largely accomplished by serological investigation using IgG and IgM ELISAs, as the requirement for species-specific conjugate is not as burdensome as in veterinary medicine. There are numerous commercial serological tests available for the detection of *Toxoplasma* exposure in humans, one study compared three ELISAs, two IHAs, one IFA and one LAT to the Center for Disease Control's reference indirect immunofluorescence test and found 100% sensitivity across the tests, with a range of specificities from 77.8-100%, and perfect agreement being noted by one representative of each testing method (Wilson *et al.*, 1987). The reliance on a single serum test (e.g. IgM ELISA) to determine the critical question of chronicity of infection, however, complicates the diagnostic picture, as false positives indicating acute infection can have dramatic consequences in

the management of patients, especially pregnant women. Several studies have noted high sensitivities but variable specificities for commercial IgM ELISAs, with a positive predictive value of only 43.3% noted in one (Kodym *et al.*, 2006; Roberts *et al.*, 2001; Wilson *et al.*, 1997). Confirmation testing must be an absolute requirement in these cases, two options being paired IgG testing or IgG avidity testing (Liesenfeld *et al.*, 1997). While few IgM ELISAs are utilized for veterinary diagnostics, IgM testing can be accomplished by utilizing modified agglutination methods, but the same cautions must be noted when relying on a single serum sample for the determination of infection chronicity.

In veterinary diagnostic investigation for *Toxoplasma*, the production of recombinant antigen and advance of molecular techniques has expanded diagnostic options, while more established agglutination techniques are becoming less commercially available. Some species specific ELISAs have been developed and performed well when compared to reference serological techniques, notably for cats, sheep and pigs (Dabritz *et al.*, 2007; Hill *et al.*, 2006; Kimbita *et al.*, 2001; Klun *et al.*, 2007; Mainar-Jaime and Barberan, 2007; Shaapan *et al.*, 2008). Some cautions that must be noted in interpretation of these results include the potential of cross-reactivity in the presence of infections with other coccidia and the difficulty of determining true sensitivities and specificities without a reliable gold standard for comparison (Gardner *et al.*, 2009; Silva *et al.*, 2002). A notable weakness for these ELISA procedures is their requirement for species-specific conjugates, which appears to require a large number of individual ELISAs to test the range of animal serum submitted to veterinary diagnostic laboratories, making them a poor replacement for the established agglutination

techniques. Some investigators have examined the use of non-species specific protein conjugates in veterinary diagnostic testing, e.g. proteins A, G and A/G, with some success (Bhide *et al.*, 2004; Eliasson *et al.*, 1989; Werre *et al.*, 2002; Zhang *et al.*, 2010), and this technique is developed further as described below. While serological detection is the primary mode of *Toxoplasma* diagnosis, molecular techniques are increasingly being used, since the first identification of a gene target for polymerase chain reaction amplification (Burg *et al.*, 1989). PCR is now commonly used to test amniotic fluid in pregnant women with suggestive serological results, and has been used for environmental detection of oocysts and characterization of *Toxoplasma* genotypes in vertebrate hosts (Schwab and McDevitt, 2003; Su *et al.*, 2006). The difficulty of acquiring the appropriate tissue sample for antemortem diagnosis makes PCR a modality of questionable sensitivity but potentially excellent specificity when used in the diagnostic setting, a question which is further examined below.

SPECIFIC AIMS

Numerous commercially produced diagnostic kits are available for evaluation of human serum for evidence of *Toxoplasma* exposure. A human IgG ELISA kit was modified by the use of either protein A, protein G or protein A/G chimeric conjugate to replace the goat anti-human IgG protein conjugate supplied with the kit to allow evaluation of nonhuman animal sera. These ELISAs were compared to previous results generated by a commercially produced agglutination test (IHA) using catalogued sera previously submitted to the New York Animal Health Diagnostic Center for *Toxoplasma* testing.

Traditional and real time PCR assays were developed based on the *B1* gene sequence and sensitivities of both were tested using cultured tachyzoites. These assays were then used to test sera with known anti-*Toxoplasma* IgG for the presence of *Toxoplasma* DNA. The modified protein A/G conjugate ELISA was used to detect seroconversion in a population of New York State hunter-killed white-tailed deer.

1. Determine efficacy of replacement of protein A or protein G conjugate in human serologic kit in testing for *Toxoplasma*
2. Test replacement of kit conjugate with chimeric protein A/G conjugate to establish a single test for animal diagnostics
3. Determine the most sensitive primer in PCR detection of *Toxoplasma* DNA and demonstrate minimum detectable copy number by both traditional and real time PCR
4. Describe risk factors for *Toxoplasma* exposure in a retrospective case-control study of New York State hunter-killed deer

BIBLIOGRAPHY

- Bhide MR, J Curlik, M Travnicek, P Lazar. 2004. Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs. *Comp Immunol Microbiol Infect Dis* 27: 191-199.
- Burg JL, CM Grover, P Pouletty, JC Boothroyd. 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain-reaction. *J Clin Microbiol* 27: 1787-1792.
- Cole RA, DS Lindsay, DK Howe, CL Roderick, JP Dubey, NJ Thomas, LA Baeten. 2000. Biological and molecular characterizations of *Toxoplasma gondii* strains obtained from southern sea otters (*Enhydra lutris nereis*). *J Parasitol* 86: 526-530.
- Cook AJC, RE Gilbert, W Buffolano, J Zufferey, E Petersen, PA Jenum, W Foulon, AE Semprini, DT Dunn on behalf of the European Research Network on Congenital Toxoplasmosis. 2000. Sources of *Toxoplasma* infection in pregnant women: European multicenter case-control study. *BMJ* 321: 142-147
- Crist SC, RL Stewart Jr., JP Rinehart, GR Needham. 1999. Surveillance for *Toxoplasma gondii* in the white-tailed deer (*Odocoileus virginianus*) in Ohio. *Ohio J Sci* 99: 34-37.
- Dabritz HA, PA Conrad. 2010. Cats and *Toxoplasma*: Implications for public health. *Zoonoses Public Health* 57: 34-52.
- Dabritz HA, IA Gardner, MA Miller, MR Lappin, ER Atwill, AE Packham, AC Melli, PA Conrad. 2007. Evaluation of two *Toxoplasma gondii* serologic tests used in a serosurvey of domestic cats in California. *J Parasitol* 93: 806-816.
- Denkers EY, AG Schneider, SB Cohen, BA Butcher. 2012. Phagocyte responses to protozoan infection and how *Toxoplasma gondii* meets the challenge. *PLoS Pathog* 8: e1002794
- Desmonts G, JS Remington. 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *J Clin Microbiol* 11: 562-568.
- deSouza SLP, SM Gennari, LEO Yai, SRN D'Auria, SMS Cardoso, JSG Junior, JP Dubey. 2003. Occurrence of *Toxoplasma gondii* antibodies in sera from dogs of the urban and rural areas from Brazil. *Rev Brasil Parasitol Vet* 12: 1-3.
- Dubey JP. 2008. The history of *Toxoplasma gondii* – the first 100 years. *J Eukaryot Microbiol* 55: 467-475.

- Dubey JP. 2010. Toxoplasmosis of animals and humans, 2nd edition. CRC Press. Boca Raton, FL.
- Dubey JP, DS Adams. 1990. Prevalence of *Toxoplasma gondii* antibodies in dairy goats from 1982 to 1984. JAVMA 196: 295-296.
- Dubey JP, CR Bhatia, MR Lappin, LR Ferreira, A Thorn, OCH Kwok. 2009. Seroprevalence of *Toxoplasma gondii* and *Bartonella* spp. antibodies in cats from Pennsylvania. J Parasitol 95: 578-580.
- Dubey JP, G Desmonts. 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet J 19: 337-339.
- Dubey JP, HR Gamble, D Hill, C Sreekumar, S Romand, P Thulliez. 2002. High prevalence of viable *Toxoplasma gondii* infection in market weight pigs from a farm in Massachusetts. J Parasitol 88: 1234-1238.
- Dubey JP, MC Jenkins, OCH Kwok, RL Zink, ML Michalski, V Ulrich, J Gill, M Cartensen, P Thulliez. 2009. Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in white tailed deer (*Odocoileus virginianus*) from Iowa and Minnesota using four serologic tests. Vet Parasitol 161: 330-334.
- Dubey JP, WJA Saville, JF Stanek, SM Reed. 2002. Prevalence of *Toxoplasma gondii* antibodies in domestic cats from rural Ohio. J Parasitol 88: 802-803
- Dubey JP, N Sundar, D Hill, GV Velmurugan, LA Bandini, OC Kwok, D Majumdar, C Su. 2008. High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA. Int J Parasitol 38: 999-1006.
- Dubey JP, P Thulliez. 1989. Serologic diagnosis of toxoplasmosis in cats fed *Toxoplasma gondii* tissue cysts. JAVMA 194: 1297-1299.
- Dubey, JP, GV Velmurugan, V Ulrich, J Gill, M Carstensen, N Sundar, OCH Kwok, P Thulliez, D Majumdar, C Su. 2008. Transplacental toxoplasmosis in naturally-infected white-tailed deer: isolation and genetic characterisation of *Toxoplasma gondii* from fetuses of different gestational ages. Int J Parasitol 38: 1057-1063.
- Dubey JP, FL Welcome. 1988. *Toxoplasma gondii*-induced abortion in sheep. JAVMA 193: 697-700.
- Eliasson M, R Andersson, A Olsson, H Wigzell, M Uhlen. 1989. Differential IgG-binding characteristics of staphylococcal protein A, streptococcal protein G, and a chimeric protein AG. J Immunol 142: 575-581.

- Ferguson DJP. 2009. *Toxoplasma gondii*: 1908-2008, homage to Nicolle, Manceaux and Splendore. Mem Inst Oswaldo Cruz 104: 133-148.
- Frenkel JK, JP Dubey, NL Miller. 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. Science 167: 893-896.
- Gamble HR, JP Dubey, DN Lambillotte. 2005. Comparison of a commercial ELISA with the modified agglutination test for detection of *Toxoplasma* infection in the domestic pig. Vet Parasitol 128: 177-181.
- Garcia-Vazques Z, R Rosario-Cruz, G Diaz-Garcia, O Hernandez-Baumgarten. 1993. Seroprevalence of *Toxoplasma gondii* infection in cattle, swine, and goats in four Mexican states. Prev Vet Med 17: 127-132.
- Gardner IA, M Greiner, JP Dubey. 2009. Statistical evaluation of test accuracy studies for *Toxoplasma gondii* in food animal intermediate hosts. Zoonoses Public Health
- Hartley WJ, SC Marshall. 1957. Toxoplasmosis as a cause of ovine perinatal mortality. NZ Vet J 5: 119-124.
- Hill DE, S Chirukandoth, JP Dubey, JK Lunney, HR Gamble. 2006. Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine. Vet Parasitol 141: 9-17.
- Hill RE, JJ Zimmerman, RW Wills, S Patton, WR Clark. 1998. Seroprevalence of antibodies against *Toxoplasma gondii* in free-ranging mammals in Iowa. J Wildl Dis 34: 811-815.
- Humphreys JG, RL Stewart, JP Dubey. 1995. Prevalence of *Toxoplasma gondii* antibodies in sera of hunter-killed white-tailed deer in Pennsylvania. Am J Vet Res 56: 172-173.
- Hutchison WM. 1965. Experimental transmission of *Toxoplasma gondii*. Nature 206: 961-962.
- Jittapalapong S, B Nimsupan, N Pinyopanuwat, W Chimnoi, H Kabeya, S Maruyama. 2007. Seroprevalence of *Toxoplasma gondii* antibodies in stray cats and dogs in the Bangkok metropolitan area, Thailand. Vet Parasitol 145: 138-141.
- Kimbita EN, X Xuan, X Huang, T Miyazawa, S Fukumoto, M Mishima, H Suzuki, C Sugimoto, H Nagasawa, K Fujisaki, N Suzuki, T Mikami, I Igarashi. 2001. Serodiagnosis of *Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay using recombinant SAG1. Vet Parasitol 102: 35-44.

- Klun I, O Djurkovic-Djakovic, P Thulliez. 2007. Comparison of a commercial ELISA with the modified agglutination test for the detection of *Toxoplasma gondii* infection in naturally exposed sheep. *Zoonoses Public Health* 54: 165-168.
- Kodym P, L Machala, H Rohacova, B Sirocka, M Maly. 2006. Evaluation of a commercial IgE ELISA in comparison with IgA and IgM ELISAs, IgG avidity assay and complement fixation for the diagnosis of acute toxoplasmosis. *Clin Microbiol Infect* 13: 40-47.
- Lappin MR, CC Powell. 1991. Comparison of latex agglutination, indirect hemagglutination, and ELISA techniques for the detection of *Toxoplasma gondii*-specific antibodies in the serum of cats. *J Vet Intern Med* 5: 299-301.
- Levaditi C, R Schoen, V Sanchis Bayarri. 1928. L'encephalo-myelite toxoplasmique chronique du lapin et de la souris. *CR Soc Biol* 99: 37-40.
- Liesenfeld O, C Press, JG Montoya, R Gill, JL Isaac-Renton, K Hedman, JS Remington. 1997. False-positive results in immunoglobulin M (IgM) *Toxoplasma* antibody tests and importance of confirmatory testing: the Platelia Toxo IgM test. *J Clin Microbiol* 35: 174-178.
- Lindsay DS, JP Dubey, SJ Upton, RK Ridley. 1990. Serological prevalence of *Neospora caninum* and *Toxoplasma gondii* in dogs from Kansas. *J Helminthol Soc Wash* 57: 86-88.
- Lindsay, DS, LM Weiss and Y Suzuki. 2003. Toxoplasmosis in "North American Parasitic Zoonoses", edited by Richardson and Krause. *World Class Parasites*, V 6: 129-150.
- Lunde MN. 1973. Laboratory methods in the diagnosis of toxoplasmosis. *Health Lab Sci* 10: 319-328.
- Luria BJ, JK Levy, MR Lappin, EB Breitschwerdt, AM Legendre, JA Hernandez, SP Gorman, IT Lee. 2004. Prevalence of infectious diseases in feral cats in northern Florida. *J Feline Med Surg* 6: 287-296
- Mainar-Jaime RC, M Barberan. 2007. Evaluation of the diagnostic accuracy of the modified agglutination test (MAT) and an indirect ELISA for the detection of serum antibodies against *Toxoplasma gondii* in sheep through Bayesian approaches. *Vet Parasitol* 148: 122-129.
- McSporran KD, C McCaughan, JHS Currall, A Demsteegt. 1985. Toxoplasmosis in goats. *NZ Vet J* 33: 39-40.

- Mead, PS, L Slutsker, V Dietz, LF McCaig, JS Bresee, C Shapiro, PM Griffin, RV Tauxe. 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5: 607-624.
- Meireles LR, AJ Galisteo Jr, E Pompeu, HF Andrade Jr. 2004. *Toxoplasma gondii* spreading in an urban area evaluated by seroprevalence in free-living cats and dogs. *Trop Med Int Health* 9: 876-881.
- Mello U. 1910. Un cas de toxoplasmose du chien observe a Turin (2). *Bull Soc Pathol Exot Fil* 3: 359-363.
- Nicolle C, L Manceaux. 1909. Sur un protozoaire nouveau du gondi. *CR Seances Acad Sci* 148: 369-372
- Olafson P, WS Monlux. 1942. *Toxoplasma* infection in animals. *Cornell Vet*, 32:76–190.
- Patton S, SS Johnson, K Puckett. 1990. Prevalence of *Toxoplasma gondii* antibodies in nine populations of dairy goats: compared titers using modified direct agglutination and indirect hemagglutination. *J Parasitol* 76: 74-77.
- Reiter-Owona I, E Petersen, D Joynson, H Aspöck, ML Darde, R Disko, O Dreazen, H Dumon, R Grillo, U Gross, M Hayde, R Holliman, DO Ho-Yen, K Janitschke, PA Jenum, K Naser, M Olszewski, P Thulliez, HM Seitz. 1999. The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. *Bull WHO* 77: 929-935.
- Roberts A, K Hedman, V Luyasu, J Zufferey, M-H Bessieres, R-M Blatz, E Candolfi, A Decoster, G Enders, U Gross, E Guy, M Hayde, D Ho-Yen, J Johnson, B Lecolier, A Naessens, H Pelloux, P Thulliez, E Petersen. 2001. Multicenter evaluation of strategies for serodiagnosis of primary infection with *Toxoplasma gondii*. *Clin Microbiol Infect Dis* 20: 467-474.
- Sabin AB, HA Feldman. 1948. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*). *Science* 108: 660-663.
- Sabin AB, PK Olitsky. 1937. *Toxoplasma* and obligate intracellular parasitism. *Science* 85: 336-338.
- Santos TR, AJ Costa, GH Toniollo, MC Luvizotto, AH Benetti, RR Santos, DH Matta, WD Lopes, JA Oliveira, GP Oliveira. 2009. Prevalence of anti-*Toxoplasma gondii* antibodies in dairy cattle, dogs, and humans from the Jauru micro-region, Mato Grosso state, Brazil. *Vet Parasitol* 161: 324-326.

- Schares G, N Pantchev, D Barutzki, AO Heydorn, C Bauer, FJ Conraths. 2005. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. *Int J Parasitol* 35: 1525-1537.
- Schares G, MG Vrhovec, N Pantchev, DC Herrmann, FJ Conraths. 2008. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. *Vet Parasitol* 152: 34-45
- Schwab DJ, JJ McDevitt. 2003. Development of a PCR-enzyme immunoassay oligoprobe detection method for *Toxoplasma gondii* oocysts, incorporating PCR controls. *Appl Environ Microbiol* 69: 5819-5825.
- Shaapan RM, FA El-Nawawi, MAA Tawfik. 2008. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Vet Parasitol* 153: 359-362.
- Shen L, L Zhichung, Z Biaucheng, Y Huayuan. 1990. Prevalence of *Toxoplasma gondii* infection in man and animals in Guangdong, Peoples' Republic of China. *Vet Parasitol* 34: 357-360
- Silva NM, EV Lourenco, DAO Silva, JR Mineo. 2002. Optimisation of cut-off titres in *Toxoplasma gondii* specific ELISA and IFAT in dog sera using immunoreactivity to SAG-1 antigen as a molecular marker of infection. *Vet J* 163: 94-98.
- Spencer JA, MJ Higginbotham, BL Blagburn. 2003. Seroprevalence of *Neospora caninum* and *Toxoplasma gondii* in captive and free-ranging nondomestic felids in the United States. *J Zoo Wildl Med* 34: 246-249.
- Splendore A. 1908. Un nuovo protozoa parassita de' conigli, incontrato nelle lesion anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell' uomo. Nota preliminare pel. *Rev Soc Scient Sao Paulo* 3: 109-112.
- Su C, X Zhang, JP Dubey. 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol* 36: 841-848.
- Vanek JA, JP Dubey, P Thulliez, MR Riggs, BE Stromberg. 1996. Prevalence of *Toxoplasma gondii* antibodies in hunter-killed white-tailed deer (*Odocoileus virginianus*) in four regions of Minnesota. *J Parasitol* 82: 41-44.
- Vollaire MR, SV Radecki, MR Lappin. 2005. Seroprevalence of *Toxoplasma gondii* antibodies in clinically ill cats in the United States. *AJVR* 66: 874-877.
- Weinman D, AH Chandler. 1954. Toxoplasmosis in swine and rodents. Reciprocal oral infection and potential human hazard. *Proc Soc Exp Biol Med* 87: 211-216.

- Wendte, JM, AK Gibson, ME Grigg. 2011. Population genetics of *Toxoplasma gondii*: new perspectives from parasite genotypes in wildlife. *Vet Parasitol* 182: 96-111.
- Werre SR, RH Jacobson, DD Bowman, JP Dubey, HO Mohammed. 2002. Evaluation of kinetics and single-read enzyme-linked immunoassays for detection of *Toxoplasma gondii* antibodies in sheep. *J Vet Diagn Invest* 14: 225-230.
- Wilson M, JS Remington, C Clavet, G Varney, C Press, D Ware and the FDA toxoplasmosis ad hoc working group. 1997. Evaluation of six commercial kits for detection of human immunoglobulin M antibodies to *Toxoplasma gondii*. *J Clin Microbiol* 35; 3112-3115.
- Wilson M, DA Ware, KW Walls. 1987. Evaluation of commercial serodiagnostic kits for toxoplasmosis. *J Clin Microbiol* 25: 2262-2265.
- Wolf A, D Cohen, B Paige. 1939. Human toxoplasmosis: occurrence in infants as an encephalomyelitis verification by transmission to animals. *Science* 89: 226-227.
- Zaki. 1995. Seroprevalence of *Toxoplasma gondii* in domestic animals in Pakistan. *J Pak Med Assoc* 45: 4-5.
- Zhang D, Z Wang, R Fang, H Nie, H Feng, Y Zhou, J Zhao. 2010, Use of protein AG in ELISA for serodiagnosis of *Toxoplasma gondii* infection in four species of animals. *Clin Vaccine Immunol* 17:485–486.

CHAPTER TWO

MODIFICATION OF A COMMERCIAL *TOXOPLASMA GONDII* IMMUNOGLOBULIN G ENZYME-LINKED IMMUNOSORBENT ASSAY FOR USE IN MULTIPLE ANIMAL SPECIES

ABSTRACT

A challenge faced by veterinary diagnosticians in serologic analysis for exposure to pathogens is the need for protein conjugate capable of antibody attachment in many animal species. The advent of protein conjugates that are less specific in nature allows diagnosis across many species with little or no modification of technique. *Toxoplasma gondii* is an organism of veterinary interest that has been demonstrated to infect a plethora of warm-blooded animals. However, the serologic tests available for simultaneous diagnosis in this broad range are limited in number. The current study examined the use of an immunoglobulin G enzyme-linked immunosorbent assay (ELISA) modified by the use of non-species-specific protein conjugates in domestic animal species commonly submitted to diagnostic laboratories for evaluation of *Toxoplasma* exposure status. Comparison to results from an established indirect hemagglutination technique revealed very good agreement between the 2 test methods. This modification of the ELISA provides a useful method for veterinary diagnosticians to perform rapid and accurate evaluation of multiple animal species for *Toxoplasma* exposure using a single test.

INTRODUCTION

Toxoplasma gondii is a protozoal organism that can cause clinical disease in both definitive and intermediate hosts and is believed to be one of the most common parasitic infections in the world (Kijlstra *et al.*, 2004). Toxoplasmosis has been described from human beings and numerous wild and domestic animals, including birds, canids, felids, rodents, and ruminants (Lindsey and Dubey, 2007). The risk of congenital transmission with potentially severe consequences for the fetus in acutely infected pregnant women has generated great public interest in this organism, as has emerging recognition of its importance in causing disease in immunocompromised patients (Montoya and Liesenfeld, 2004). In France, concern about congenital toxoplasmosis is such that pregnant women are tested monthly to detect the presence of recent infection (Remington *et al.*, 2004). Felids have been demonstrated to be the definitive host for the organism, while a great diversity of vertebrates, including human beings and many domestic and wild animals, have been found to be intermediate hosts.

Infection of the definitive felid host can be acquired through predation or scavenging of meat from an infected intermediate host via tissue cysts, by ingestion of oocysts shed by an infected felid, or by congenital transmission from an infected queen. Infection is established in the intestinal tract where asexual and sexual reproduction takes place, leading to fecal shedding of oocysts, potentially accompanied by more generalized infection. Intermediate hosts can similarly be infected by either ingestion of oocysts or tissue cysts. In intermediate and dead-end hosts, sporozoites from ingested oocysts or bradyzoites from ingested tissue cysts enter the wall of the small intestine. Tachyzoite development leads to systemic spread and invasion of numerous organs

where bradyzoite-containing tissue cyst development proceeds (Dubey, 2004; Ferguson and Dubremetz, 2007; Mucker et al., 2006; Speer and Dubey, 1998). The complexity of the life cycle of *Toxoplasma* and the relatively short period of oocyst shedding from the definitive host has led to the development of numerous serologic techniques to diagnose infection. The ideal serologic test for diagnosis in the veterinary setting is objective, has high throughput potential, and demonstrates both high sensitivity and specificity in numerous species.

Clinical signs in definitive and intermediate hosts due to infection with *Toxoplasma* vary in severity from negligible to terminal disease. A great deal of concern in both human and animal health involves the risk of abortions and fetal malformations in the presence of acute infection. Among domestic animals, abortion in sheep and goats is widely noted, while significance in cattle is less well defined, likely due to the similarity to manifestation of *Neospora caninum* infection (Dubey and Lindsay, 1996). Most cats are believed to remain asymptomatic upon initial infection, though some display nonspecific signs such as fever, dyspnea, and abdominal pain (Dubey and Carpenter, 1993). Space-occupying lesions created by enlarging tissue cysts can produce disease, related to their site of localization in the vertebrate intermediate host, notably causing neurologic and/or ocular deficits.

The relatively brief period of oocyst shedding in the definitive felid host, combined with the persistent immunoglobulin G (IgG) antibody response in both definitive and intermediate host infections, make serologic evaluation the method of choice for detecting exposure to *Toxoplasma* in all potential host species (Dabritz and Conrad, 2010; Dabritz et al., 2007; Dubey, 1995; Dubey, 2001; Dubey, 2004; Dubey et al.,

1977). Techniques that have been commonly used include the Sabin–Feldman dye test, latex agglutination, hemagglutination assays, fluorescent antibody tests (FATs) and enzyme-linked immunosorbent assays (ELISAs). Some of these are hampered in the veterinary diagnostic environment by the use of live organism (Sabin–Feldman) or by the requirement for species-specific conjugate (FAT and ELISA). In the current study, the results of a commercially produced indirect hemagglutination assay were compared with those of a commercially available IgG ELISA, modified to detect IgG antibody in multiple animal species.

MATERIALS AND METHODS

Animals and samples

Blood samples were collected from animals by referring veterinarians into clot tubes. The extracted serum samples were submitted to the Animal Health Diagnostic Center at Cornell University (Ithaca, New York) from 2001 to 2008 for detection of *Toxoplasma* exposure by indirect hemagglutination. After storage at 5°C, positive samples with a sufficient amount of serum remaining and a sampling of negative samples were reassessed using the modified IgG ELISA technique in 2008–2009. The sera tested included samples from 46 alpacas, 26 cats, 53 dogs, 39 goats, 21 horses, and 42 sheep.

Indirect hemagglutination assay

Seroreactivity to *Toxoplasma* antigens was initially evaluated using an indirect hemagglutination assay (IHA).^a The commercial preparation of the IHA had perfect qualitative agreement with the Center for Disease Control and Prevention (CDC)

Toxoplasma indirect FAT in their evaluation of human serum samples (Wilson *et al.*, 1987). Serum containing anti-*Toxoplasma* IgG antibody causes agglutination of the sheep red blood cells coated with antigen as part of the test procedure (Chordi *et al.*, 1964; Jacobs and Lunde, 1957). The test procedure distinguishes these reactions from nonspecific agglutination through the use of both coated and non-coated red blood cells. Interpretation is based on observation of presence or absence of a red blood cell mat covering the bottom of the test well on completion of incubation. The test is equally effective for human and animal diagnostic work, as no species-specific conjugate is required for antibody detection (Wilson and McAuley, 1991). This procedure was used as a reference test to evaluate the performance of the modified IgG ELISA.

The IHA test was prepared by adding 25 µl of sample diluent (buffered saline containing 0.1% sodium azide and 1% normal rabbit serum) to the bottom of as many U-shaped wells on a 96-well microdilution test tray as will be required and adding an equal volume of the liquid to be tested. Two rows were used to test the reactivity of the negative control (normal human serum) and 2 were used to test the reactivity of the positive control (anti-*Toxoplasma* human serum or globulin). Two rows were reserved for each sample to be tested. Sample diluent was tested for agglutination. The negative control was tested for agglutination at ratios of 1:32 and 1:64. The positive control was tested at ratios of 1:32 – 1:4,096 dilutions. Samples were initially screened from 1:32 to 1:256, and were tested at higher dilutions if positive at all of these dilutions. Sheep red blood cells sensitized with a *Toxoplasma* extract were added to 1 row of each control and sample group. Unsensitized sheep red blood cells were added to the other row. The plate was shaken on a vibrating shaker briefly, and then allowed to incubate at

room temperature for 3 hr. A positive result was indicated by the formation of a smooth mat of cells covering the bottom of the well, with no such reaction noted in the unsensitized row. The latter reaction indicates the presence of nonspecific agglutinins, and invalidates the test. A negative result produces a collection of red blood cells in a small group in the center of the bottom of the well. Serum samples that produced agglutination in the 1:64 well were considered positive for anti-*Toxoplasma* IgG antibody.

Enzyme-linked immunosorbent assay

A commercial IgG ELISA^b was modified by use of protein A^c or protein G conjugate^d to replace the conjugated goat anti-human IgG included in the kit. A similar method was used to create an ELISA for detection of anti-*Toxoplasma* antibody in sheep samples using protein G (Werre *et al.*, 2002). Samples, controls, and a calibrator were evaluated after dilution 1:21 with kit-supplied diluent (containing Tween-20, bovine serum albumin, and phosphate buffered saline). These were added to kit wells that had been sensitized by passive absorption with *Toxoplasma* antigen. Any antigen-specific antibody contained in the sample binds to the immobilized antigen. Protein A or G conjugate, depending on vertebrate species from which the sample was collected, was added to each well to attach to any immobilized IgG antibody. Protein A conjugate was used for canine and feline samples, whereas protein G conjugate was used for the remaining species. Following washing, microwells were then incubated with peroxidase substrate solution, which will change color when hydrolyzed by the presence of peroxidase. Color intensity of the sample indicated antibody concentration in the microwell. This was evaluated using a microplate reader with light at a wavelength of

450 nm. Comparison to kit-provided calibrators guided the identification of positive versus negative serum, with a ratio of sample color intensity to kit calibrator color intensity of greater than or equal to 1.1 indicating a positive reaction for anti-*Toxoplasma* IgG antibody as described by the kit insert. A ratio between 0.9 and 1.1 (non-inclusive) was considered an indeterminate result, and less than or equal to 0.9 a negative result.

Protein A and protein G conjugates

Protein A and protein G horseradish peroxidase conjugates were used in the IgG ELISA assays instead of the kit conjugate to allow testing of multiple animal species. Protein A conjugates are reported to provide strong binding to antibody of cats, dogs, mice, pigs, and rabbits, whereas protein G conjugates are reported to provide strong binding to IgG antibody of cows, goats, guinea pigs, horses, mice, pigs, rabbits, and sheep (Eliasson *et al.*, 1988).^d Protein G conjugate also showed strong binding to alpaca antibody on testing with this procedure (unpublished data). Both proteins were received as lyophilized samples and were diluted 1:20,000 with phosphate buffered saline (pH 7.2) prior to use, with the dilution determined by testing against kit controls to satisfy quality control parameters. Similar methods have been used with these proteins and a chimera of the 2 proteins in previous studies (Bhide *et al.*, 2004; Surolia *et al.*, 1982; Zhang *et al.*, 2010).

Data analysis

The significance of association between the results of the IHA and the ELISA was evaluated using the McNemar Chi-square test. The degree of agreement between the results from the 2 tests was quantified using Kappa statistics. The accuracy of the

ELISA test in detecting exposure to *Toxoplasma* was evaluated in comparison to the IHA and measured using the relative sensitivity and specificity. The expected performance of the ELISA at different cut-off points was examined using the receiver operating characteristic (ROC) curves for all data and additionally separated by animal species.

RESULTS

Serum samples were evaluated from multiple animal species using both the IHA and the IgG ELISA as described. The protein G conjugate replacement was used to analyze alpaca, caprine, equine, and ovine sera. Protein A conjugate was used to analyze canine and feline sera. The sera tested included samples from 46 alpacas, 26 cats, 53 dogs, 39 goats, 21 horses, and 42 sheep. There was no significant difference detected between positive versus negative results when comparing IHA and ELISA results with McNemar's Chi-square ($p = .07$). The associated kappa value using the kit standard cut-off parameters was 0.83 (95% confidence interval [CI]: 0.75–0.90 calculated using GraphPad[®]), indicating very good agreement between the 2 test results. The combined sensitivity of the protein A/G modified ELISA kit was found to be 90% (95% CI: 83–94%) and specificity 95% (95% CI: 87–98%) when the IHA results were utilized as a gold standard and positive versus negative ELISA results were determined using the manufacturer's guidelines. It is noted that adjusting the range of indeterminate results to 0.8–1.0 ratios improves sensitivity based on this data to 92% from 90%, while specificity remains at 95% (Figs. 2.1, 2.2), and should be considered for animal samples. The prevalence of positives in the samples tested was 59% (Table

2.1), at a prevalence of 10% (the approximate fraction of positive samples submitted to the Animal Health Diagnostic Center based on IHA results), the calculated positive predictive value would be 67% with a negative predictive value of 99%. Table 2.2 documents the range of test performances by species using the kit-recommended parameters for distinguishing positive from negative results. In addition, ROC curves are provided for all results collectively and by species to evaluate the ELISA's performance when compared to the IHA (Figs. 2.1–2.3).

DISCUSSION

Antemortem diagnosis of toxoplasmosis presents a challenge due to the nonspecific nature or lack of clinical signs. Serologic evaluation provides a useful tool by the ease of access to the requisite sample and its high sensitivity to detection of recent and chronic infection. However, care must be used in linking an animal's serologic status to active illness due to this same ability to detect historic infection. The cessation of production of a commercially available IHA kit in the United States has led to a critical gap in diagnostic options for serologic diagnosis of *Toxoplasma* infection across a wide range of animal species, notably intermediate hosts like goats and sheep in which toxoplasmosis can cause significant economic losses. The use of ELISAs for similar detection has been limited by the requirement for species-specific anti-IgG conjugate to produce valid results. The successful testing of an ELISA that uses conjugates effective across many of the animal species of interest provides an additional tool for veterinary diagnostic laboratories to use in determining an animal's serological status.

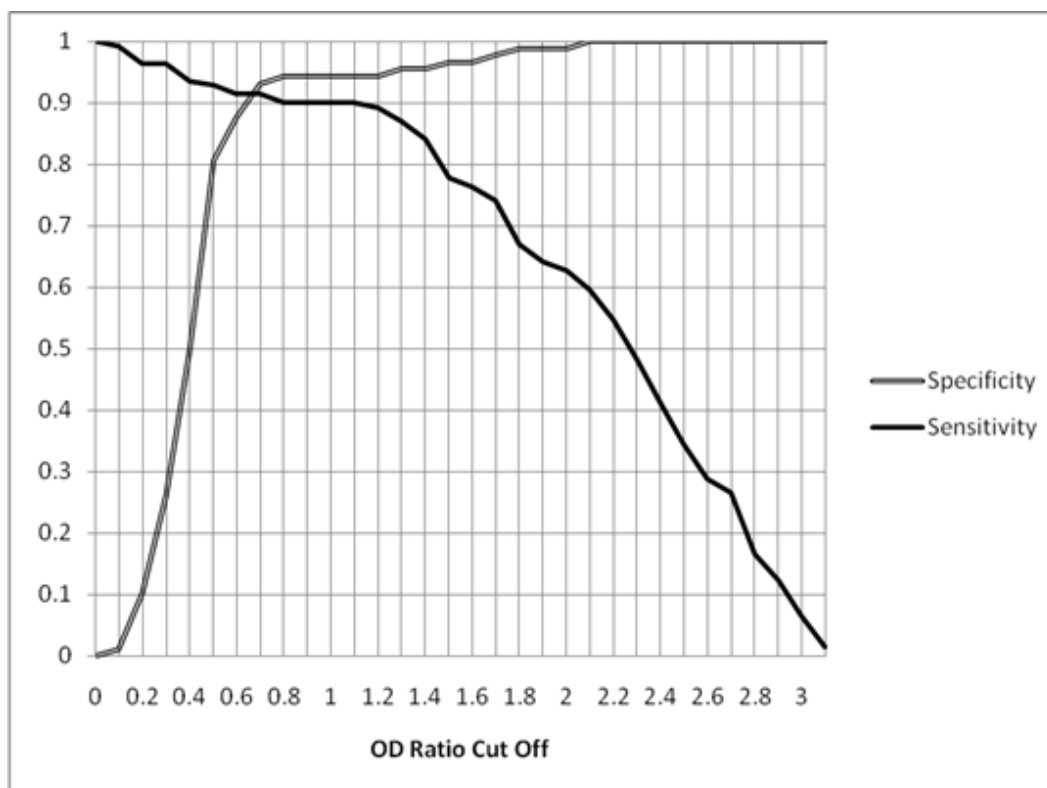


Figure 2.1. Effect of enzyme-linked immunosorbent assay optical density (OD) ratio cut-off value on relative sensitivity and specificity.

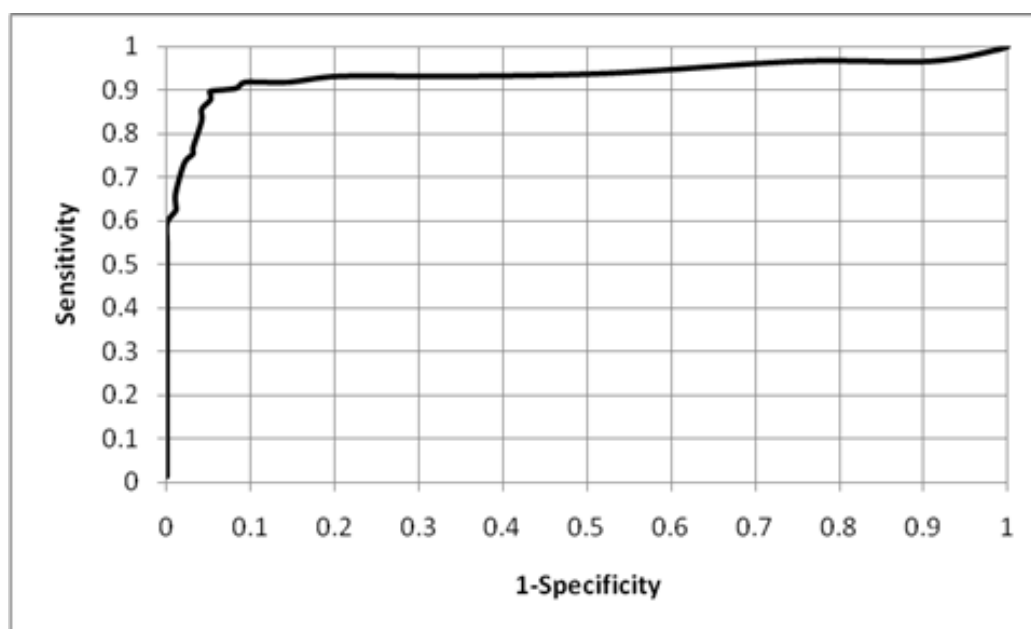


Figure 2.2. Receiver operating characteristic calculation for modified immunoglobulin G enzyme-linked immunosorbent assay.

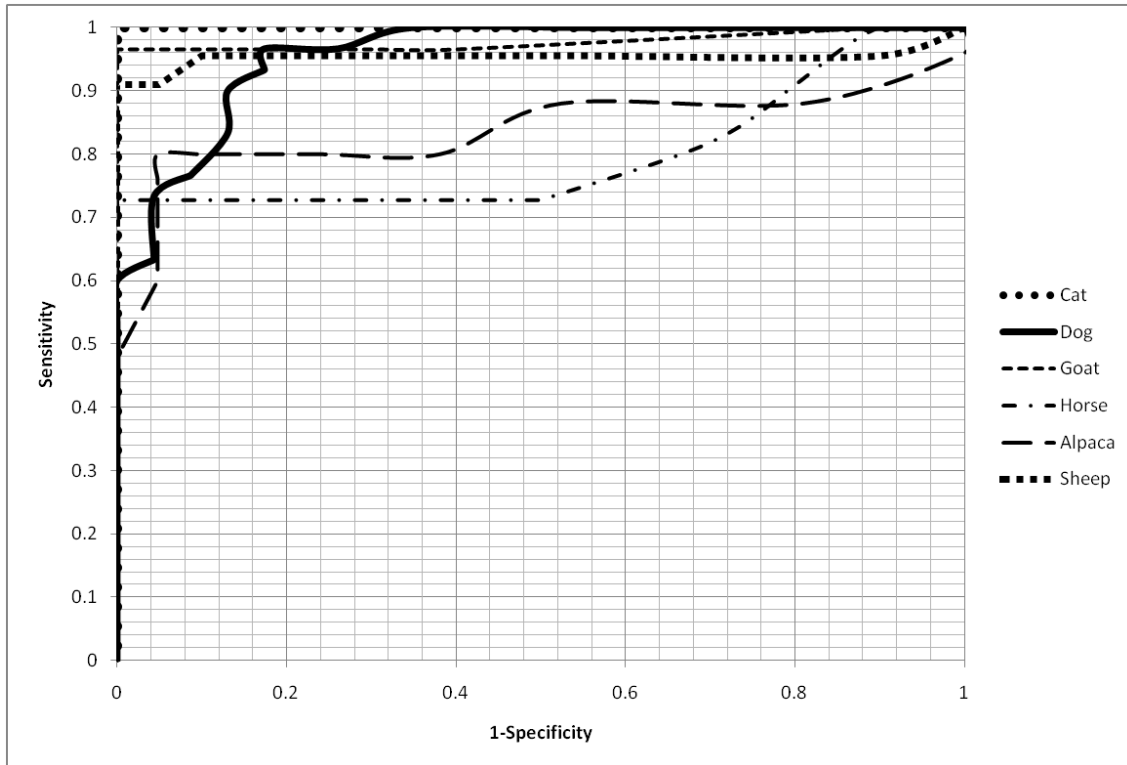


Figure 2.3. Receiver operating characteristic calculation by species

Table 2.1. 2 × 2 comparison of indirect hemagglutination assay (IHA) to modified immunoglobulin G enzyme-linked immunosorbent assay (ELISA) testing.

	ELISA+	ELISA–	Total
IHA+	125	14	139
IHA–	5	83	88
Total	130	97	227

Table 2.2. Kappa value calculation by species. *

Species	Serum samples	Kappa value (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
All tested	227	0.83(0.75–0.90)	0.90(0.83–0.94)	0.95(0.87–0.98)
<i>Canis familiaris</i>	53	0.77 (0.59–0.94)	0.93 (0.76–0.99)	0.82 (0.60–0.94)
<i>Capra hircus</i>	39	0.94 (0.81–1.0)	0.97 (0.80–1.0)	1.0 (0.66–1.0)
<i>Equus caballus</i>	21	0.72 (0.42–1.0)	0.72 (0.39–0.93)	1.0 (0.66–1.0)
<i>Felis domesticus</i>	26	1.0 (0.57–1.0)	1.0 (0.82–1.0)	1.0 (0.40–1.0)
<i>Lama pacos</i>	46	0.70 (0.49–0.90)	0.76 (0.54–0.90)	0.95 (0.74–1.0)
<i>Ovis aries</i>	42	0.91 (0.77–1.0)	0.91 (0.69–0.98)	1.0 (0.80–1.0)

* CI = confidence interval

Both the previously available IHA and the currently available IgG ELISA allow detection of IgG in serum samples, and both provide an antigen substrate to which antibody present in the serum will attach. Beyond that, the 2 procedures utilize quite different technology to quantify antibody in a given sample. The IHA uses antigen-coated red blood cells to which antibody attaches for detection, while the ELISA uses U-shaped wells coated with antigen. The IHA results depend on the subjective determination by the operator of the extent of agglutination in an individual reaction to establish a maximum positive dilution. The interpretation of ELISA results do not depend on subjective evaluation by the operator, as the optical density in the reaction well is quantified by a microtiter plate reader.

In the present experiment, 227 serological samples from diverse animal species were evaluated pairwise for the presence of anti-*Toxoplasma* IgG antibody both by the IHA and the IgG ELISA modified with protein A or G techniques. Results indicated very good agreement between the 2 tests in determining positive versus negative samples, indicated by a kappa value of 0.83. While good agreement is also noted as indicated by kappa values for results in each species, evaluation of horse and alpaca samples showed lower sensitivities than those of other species (0.72 and 0.76, respectively), which should be noted in future use of this method. The modified ELISA described herein provides a strong alternative for veterinary diagnostic laboratories in detecting exposure to *Toxoplasma* by serologic evaluation across a range of commonly tested animal species.

Acknowledgements

The authors thank Linda Baitman (Wampole Laboratories) for assistance in securing the requisite materials for the study; and Dr. Bettina Wagner and Aziza Solomon for providing feline serum samples. This research was made possible by the USDA federal formula fund grant NYC-478430.

Sources and manufacturers

- a. Toxoplasmosis TPM-Test indirect hemagglutination kit, Wampole Laboratories, Princeton, NJ.
- b. Toxo IgG II ELISA kit, Wampole Laboratories, Princeton, NJ.
- c. Protein A-peroxidase from *Staphylococcus aureus*/horseradish, Sigma-Aldrich, St. Louis, MO.
- d. Protein G horseradish peroxidase conjugate, Molecular Probes, Invitrogen Corp., Carlsbad, CA
- e. GraphPad Software Inc., La Jolla, CA.

REFERENCES

- Bhide MR, Curlik J, Travnicek M, Lazar P: 2004, Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs. *Comp Immunol Microbiol Infect Dis* 27:191–199.
- Chordi A, Walls KW, Kagan IG: 1964, Studies on the specificity of the indirect hemagglutination test for toxoplasmosis. *J Immunol* 93:1024–1033.
- Dabritz HA, Conrad PA: 2010, Cats and *Toxoplasma*: implications for public health. *Zoonoses Public Health* 57:34–52.
- Dabritz HA, Miller MA, Atwill ER, *et al.*: 2007, Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *J Am Vet Med Assoc* 231:1676–1684.
- Dubey JP: 1995, Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *J Parasitol* 81:410–415.
- Dubey JP: 2001, Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. *J Parasitol* 87:215–219.
- Dubey JP: 2004, Toxoplasmosis—a waterborne zoonosis. *Vet Parasitol* 126:57–72.
- Dubey JP, Carpenter JL: 1993, Histologically confirmed clinical toxoplasmosis in cats: 100 cases (1952–1990). *J Am Vet Med Assoc* 203:1556–1566.
- Dubey JP, Christie E, Pappas PW: 1977, Characterization of *Toxoplasma gondii* from the feces of naturally infected cats. *J Infect Dis* 136:432–435.
- Dubey JP, Lindsay DS: 1996, A review of *Neospora caninum* and neosporosis. *Vet Parasitol* 67:1–59.
- Dubey JP, Lindsay DS, Speer CA: 1998, Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11:267–299.
- Eliasson M, Olsson A, Palmcrantz E, *et al.*: 1988, Chimeric IgG-binding receptors engineered from Staphylococcal Protein A and Streptococcal Protein G. *J Biol Chem* 263: 4323–4327.
- Ferguson DJP, Dubremetz JF: 2007, The ultrastructure of *Toxoplasma gondii*. In: *Toxoplasma gondii—the model apicomplexan: perspectives and methods*, ed. LM Weiss, Kim K, 1st ed., pp. 19–46. Academic Press (Elsevier), London, UK.

- Jacobs L, Lunde MN: 1957, A hemagglutination test for toxoplasmosis. *J Parasitol* 43:308-314..
- Kijlstra A, Meerburg BG, Mul MF: 2004, Animal-friendly production systems may cause re-emergence of *Toxoplasma gondii*. *NJAS* 52:119–132.
- Lindsay DS, Dubey JP: 2007, Toxoplasmosis in wild and domestic animals. *In: Toxoplasma gondii—the model apicomplexan: perspectives and methods*, ed. LM Weiss, Kim K, 1st ed., pp. 133–147. Academic Press (Elsevier), London, UK.
- Montoya JG, Liesenfeld O: 2004, Toxoplasmosis. *Lancet* 363:1965–1976.
- Mucker EM, Dubey JP, Lovello MJ, Humphreys JG: 2006, Seroprevalence of antibodies to *Toxoplasma gondii* in the Pennsylvania bobcat (*Lynx rufus rufus*). *J Wildl Dis* 42:188–191.
- Remington JS, Thulliez P, Montoya JG: 2004, Recent developments for diagnosis of toxoplasmosis. *J Clin Microbiol* 42:941–945.
- Speer CA, Dubey JP: 1998, Ultrastructure of early stages of infections in mice fed *Toxoplasma gondii* oocysts. *Parasitology* 116:35–42.
- Surolia A, Pain D, Khan MI: 1982, Protein A: nature's universal anti-antibody. *Trends Biochem Sci* 7:74–76.
- Werre SR, Jacobson RH, Bowman DD, et al.: 2002, Evaluation of kinetics and single-read enzyme-linked immunoassays for detection of *Toxoplasma gondii* antibodies in sheep. *J Vet Diagn Invest* 14:225–230.
- Wilson M, McAuley JB: 1991, Laboratory diagnosis of toxoplasmosis. *Clin Lab Med* 11:923–939.
- Wilson M, Ware DA, Walls KW: 1987, Evaluation of commercial serodiagnostic kits for toxoplasmosis. *J Clin Microbiol* 25:2262–2265.
- Zhang D, Wang Z, Fang R, et al.: 2010, Use of protein AG in ELISA for serodiagnosis of *Toxoplasma gondii* infection in four species of animals. *Clin Vaccine Immunol* 17:485–486.

CHAPTER THREE

CHIMERIC PROTEIN A/G CONJUGATE FOR DETECTION OF ANTI-*TOXOPLASMA GONDII* IMMUNOGLOBULIN G IN MULTIPLE ANIMAL SPECIES

ABSTRACT

Serological testing for toxoplasmosis diagnosis remains the method of choice in human medicine due to the accessibility of the requisite sample, the difficulty in predicting the parasite's location in the host for direct detection and the availability of established commercial methods. In veterinary medicine, though the first two conditions are unchanged, there is a need for commercially produced test methods that are validated for *Toxoplasma gondii* detection across the range of animal species that can serve as intermediate hosts. The development of a serological method for animals would allow the diagnosis of toxoplasmosis in individual animals and a higher throughput method for population level toxoplasmosis surveys. The incorporation of a non-species specific chimeric protein A/G conjugate into an anti-*Toxoplasma* IgG enzyme linked immunosorbent assay (ELISA) is described. Serum from potential intermediate hosts was reevaluated using this method and compared to earlier testing using an established agglutination procedure. Very good agreement between the two tests was noted ($\kappa = 0.81$), establishing the method as a useful option for veterinary diagnostic testing.

INTRODUCTION

Toxoplasmosis, caused by *Toxoplasma gondii*, is a disease that has health and economic impacts across a broad spectrum of animal species (Dubey and Jones, 2008; Kijlstra et al., 2004; Lindsay and Dubey, 2007). Diagnosis of toxoplasmosis is complicated both by the non-specific nature of clinical signs and the complexity of the pathogen's life cycle making direct detection unfeasible in most situations (Dubey, 1995; Dubey, 2004; Dubey et al., 1977; Ferguson and Dubremetz, 2007; Speer and Dubey). There are a range of serological options that have been developed to detect exposure to *Toxoplasma* in humans, some of which have utility in veterinary diagnostics (Montoya and Liesenfeld, 2004; Remington *et al.*, 2004). Various permutations of agglutination testing can be applied successfully to testing a wide range of animal species, as they have no species specific conjugate requirement (Chordi et al., 1964; Desmonts and Remington, 1980; Jacobs and Lunde, 1957). However, there are no commercially produced agglutination tests currently available in the United States, whereas many other serological tests are either available or being developed for human testing (Remington *et al.*, 2004). Unfortunately, many of these tests require protein conjugate for the detection of anti-*Toxoplasma* antibody in the serum of interest. Most of the protein conjugates available are only effective in binding to antibody from specific animal species. The description of the use of protein conjugates that bind to the IgG of many different animal species is a promising new platform for serological test development (Schaefer *et al.*, 2011; Werre *et al.*, 2002; Zhang *et al.*, 2010) .

The use of non-species-specific protein conjugates A and G allows the testing of sera from most of the veterinary species of interest on an ELISA platform. However,

this requires two parallel tests to be run with two sets of control, one for samples to be tested with protein A conjugate and one for those to be tested with protein G. The development of chimeric protein A/G conjugate from Staphylococcal protein A and Streptococcal protein G has been described (Eliasson *et al.*, 1988). This chimeric protein A/G conjugate allows the testing of all species formerly tested by the two proteins individually with a single conjugate, thereby simplifying the procedure to be more attractive as a potential substitute for currently utilized agglutination techniques (Bhide *et al.*, 2004; Zhang *et al.*, 2010). A single test allows higher throughput and is better suited to commercial development and automation. In this study, the results of a commercially produced indirect hemagglutination assay were compared with those of a commercially available IgG ELISA, modified to detect IgG antibody in multiple animal species by the substitution of protein A/G horseradish peroxidase conjugate for the kit provided anti-human conjugate.

MATERIALS AND METHODS

Blood samples were collected in clot tubes from animals by referring veterinarians. The extracted serum samples were submitted to the Animal Health Diagnostic Center at Cornell University (Ithaca, New York) from 2002 to 2008 for detection of *Toxoplasma* exposure by indirect hemagglutination. After storage at 5°C, positive samples with a sufficient amount of serum remaining and a sampling of negative samples were reassessed using the modified IgG ELISA technique in 2011. The sera tested included samples from 14 alpacas, 17 sheep, 17 goats, 15 horses, and 56 dogs.

Seroreactivity to *Toxoplasma* antigens was initially evaluated using an indirect hemagglutination assay (IHA).^a The commercial preparation of the IHA had perfect qualitative agreement with the Center for Disease Control and Prevention (CDC) *Toxoplasma* indirect immunofluorescence test in their evaluation of human serum samples (Wilson *et al.*, 1987). Serum containing anti-*Toxoplasma* antibody causes agglutination of the sheep red blood cells coated with antigen as part of the test procedure (Chordi *et al.*, 1964; Jacobs and Lunde, 1957). Nonspecific agglutination is detected by assaying coated and non-coated red blood cells in separate wells. Interpretation is based on observation of presence or absence of a red blood cell mat covering the bottom of the test well on completion of incubation. The test is equally effective for human and animal diagnostic work since no species-specific conjugate is required for antibody detection (Wilson and McAuley, 1991). This procedure was used as a reference test to evaluate the performance of the modified IgG ELISA, with details described in an earlier study (Schaefer *et al.*, 2011).

A commercial IgG ELISA^b was modified by use of protein A/G horseradish peroxidase conjugate^c to replace the conjugated goat anti-human IgG included in the kit. Samples, controls, and a calibrator were evaluated after dilution 1:21 with kit-supplied diluent (containing Tween-20, bovine serum albumin, and phosphate buffered saline). These were added to kit wells that had been sensitized by passive absorption with *Toxoplasma* antigen. Any antigen-specific antibody contained in the sample binds to the immobilized antigen. Protein A/G conjugate was added to each well to attach to any immobilized IgG antibody. Following washing, microwells were incubated with tetramethylbenzidine, a peroxidase substrate solution that changes color when

hydrolyzed by the presence of peroxidase. Color intensity of the sample indicated antibody concentration in the microwell. This was evaluated using a microplate reader with light at a wavelength of 450 nm. Comparison to kit-provided calibrators guided the identification of positive versus negative serum, with a ratio of sample color intensity to kit calibrator color intensity of greater than or equal to 1.1 indicating a positive reaction for anti-*Toxoplasma* IgG antibody as described by the kit insert. A ratio between 0.9 and 1.1 (non-inclusive) was considered an indeterminate result, and less than or equal to 0.9 was considered a negative result.

Protein A/G chimeric horseradish peroxidase conjugate was used in the IgG ELISA assays instead of the kit conjugate to allow testing of multiple animal species. This protein is produced by gene fusion of the Fc-binding domains of protein A and protein G, bacterial cell wall proteins with affinity for IgG in a range of species. It contains four Fc-binding domains from protein A and two from protein G, providing binding for all antibody species and subclasses recognized by either protein A or protein G (Eliasson *et al.*, 1988). Protein A conjugate is reported to provide strong binding to IgG antibody of mice, rabbits, cats, dogs, and pigs, whereas protein G conjugate is reported to provide strong binding to IgG antibody of mice, guinea pigs, rabbits, cows, sheep, goats, alpacas, horses, and pigs (Bjorck and Kronvall, 1984; Schaefer *et al.*, 2011; Surolia *et al.*, 1982). Protein was received as lyophilized powder and was diluted 1:40,000 with phosphate buffered saline (pH 7.2) prior to use. The dilution was determined by testing against kit controls to satisfy quality control parameters.

The significance of association between the results of the IHA and the ELISA was evaluated using the McNemar's Chi-square test (McNemar, 1947). The degree of

agreement between the results from the two tests was quantified using Kappa statistics. The accuracy of the ELISA test in detecting exposure to *Toxoplasma* was evaluated in comparison to the IHA and measured using the relative sensitivity and specificity. The expected performance of the ELISA at different cut-off points was examined using the receiver operating characteristic (ROC) curve (Greiner *et al.*, 2000).

RESULTS

Serum samples were evaluated from multiple animal species using both the IHA and the IgG ELISA modified as described. The sera tested included samples from some of the more commonly tested intermediate hosts for the parasite. There was no significant difference detected between positive versus negative results when comparing IHA and ELISA results with McNemar's Chi-square ($p=1.0$), with a kappa value of agreement of 0.81 (95% confidence interval [CI]: 0.71-0.92 calculated using GraphPad^d), as noted in Table 3.1. This reaffirms the finding of our earlier study using protein A and G conjugates individually of very good agreement between the agglutination and ELISA testing. The breakdown of these results by animal species is provided in Table 3.2, with all species, except horses, demonstrating very good to perfect agreement between the two test methods. The precision of the assay was determined to be very good, with a Kappa value of 0.90 (95% CI: 0.81-1.00) found when repeated assays were performed using the same serum samples (Table 3.3). The relative sensitivity of this ELISA using chimeric protein A/G conjugate was found to be 92% (95% CI: 82-97%) and relative specificity 89% (95% CI: 77-96% calculated using VassarStats^e) when the IHA results were utilized as a gold standard and positive versus

negative ELISA results were determined using the manufacturer's guidelines^b, including 0.9 to 1.1 as the equivocal range for optical density ratios. The variation of relative sensitivity and specificity at different optical density ratios is shown in Figure 3.1. The associated Receiver Operating Characteristic (ROC) curve is depicted in Figure 3.2.

DISCUSSION

The use of chimeric protein A/G conjugate has previously been demonstrated to be an effective means of serological testing in domestic animals (Bhide *et al.*, 2004). An earlier study explored its use for diagnostic testing in goats, dogs, cats, and pigs using an ELISA method developed in the researchers' laboratory (Zhang *et al.*, 2010). In this study, a commercially produced IgG ELISA kit for the diagnosis of *Toxoplasma* in humans was modified to broaden the application of the test to domestic animals that can act as intermediate hosts of the parasite, having the benefit of being easily available to veterinary diagnosticians. Across the species tested, the modified kit produced a relative sensitivity of 92% and specificity of 89% using kit standards for determination of positive, equivocal and negative tests when the IHA was used as a gold standard. At the current time, there are no commercially available tests in the United States for the diagnosis of toxoplasmosis that can be used across the broad spectrum of animal species that can be affected. This project demonstrates that a human ELISA kit can be modified with the replacement of a non-species specific protein conjugate for the supplied anti-human IgG protein conjugate, and produce very good results for some of the more commonly tested animal species. Non-species specific protein conjugates,

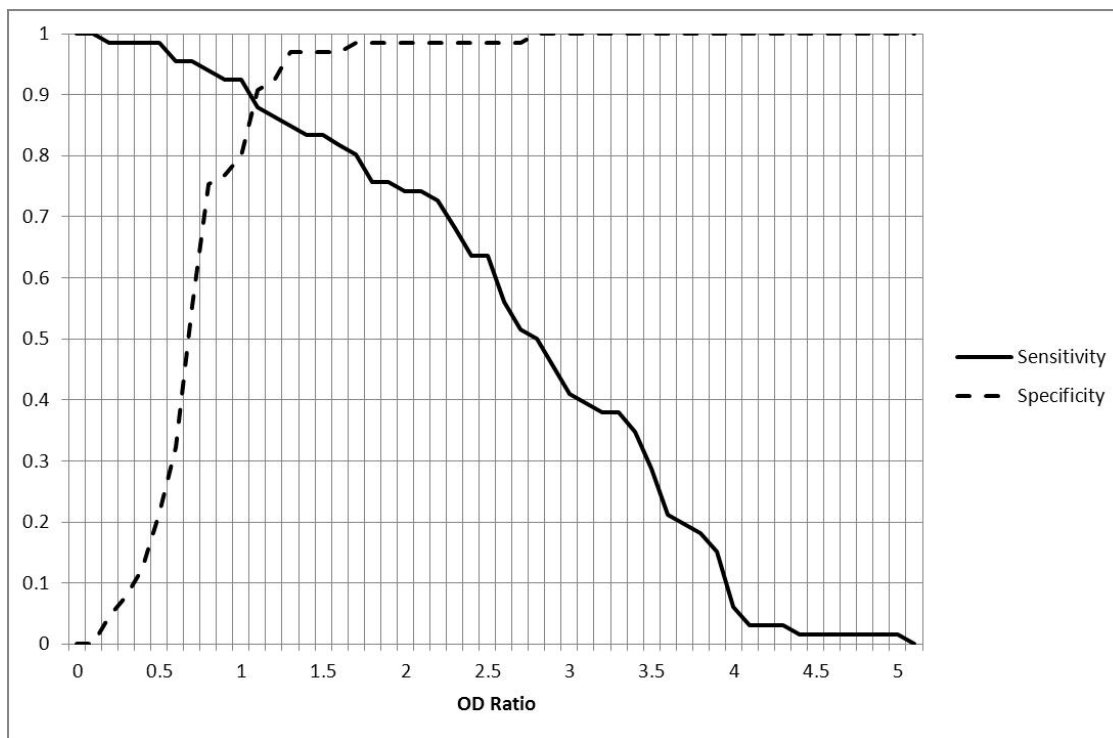


Figure 3.1. Variation in sensitivity and specificity relative to enzyme-linked immunosorbent assay optical density (OD) ratios.

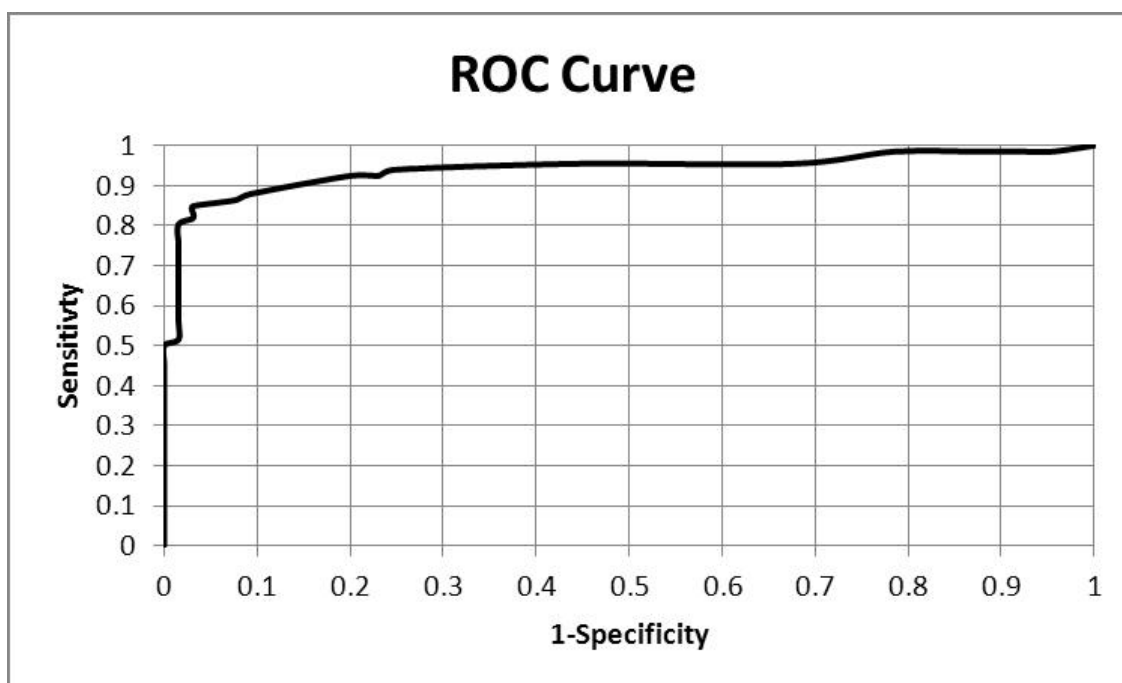


Figure 3.2. Receiver operating characteristic calculation for modified immunoglobulin G enzyme-linked immunosorbent assay.

Table 3.1. Comparison of indirect hemagglutination to modified IgG ELISA.

	ELISA +	ELISA -	Total
IHA +	58	5	63
IHA -	6	50	56
Total	64	55	119

Table 3.2. Results of modified IgG ELISA testing by species.

Species	Samples Tested	True Positive	False Positive	False Negative	True Negative	Kappa Value
Alpacas	14	8	1	0	5	0.85
Sheep	17	10	0	0	7	1.0
Goats	17	11	0	0	6	1.0
Horses	15	2	1	4	8	0.24
Dogs	56	27	4	1	24	0.82

Table 3.3. Comparison of repeated assays using modified IgG ELISA

	First Run +	First Run -	Total
Second Run +	47	2	49
Second Run -	2	33	35
Total	49	35	84

like protein A/G, have great utility in developing new veterinary diagnostic testing methods for zoonotic diseases in which there are only medical diagnostic methods available.

Acknowledgements

This research was made possible by the USDA federal formula fund grant NYC-478430.

Sources and manufacturers

- a) Toxoplasmosis TPM-Test indirect hemagglutination kit, Wampole Laboratories, Princeton, NJ.
- b) Toxo IgG II ELISA kit, Wampole Laboratories, Princeton, NJ.
- c) Recombinant protein A/G horseradish peroxidase conjugate, Thermo Fisher Scientific, Waltham, MA
- d) GraphPad Software Inc., La Jolla, CA
- e) VassarStats: Website for Statistical Computation

REFERENCES

- Bhide MR, Curlik J, Travnicek M, Lazar P: 2004, Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs. *Comp Immunol Microbiol Infect Dis* 27:191–199.
- Bjorck L, Kronvall, G: 1984, Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* 133: 969-974.
- Chordi A, Walls KW, Kagan IG: 1964, Studies on the specificity of the indirect hemagglutination test for toxoplasmosis. *J Immunol* 93:1024–1033.
- Desmonts, D, Remington JS: 1980, Direct agglutination test for diagnosis of *Toxoplasma* infection for increasing sensitivity and specificity. *J Clin Microbiol* 1:562-568.
- Dubey JP: 1995, Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *J Parasitol* 81:410–415.
- Dubey JP: 2004, Toxoplasmosis—a waterborne zoonosis. *Vet Parasitol* 126:57–72.
- Dubey JP, Christie E, Pappas PW: 1977, Characterization of *Toxoplasma gondii* from the feces of naturally infected cats. *J Infect Dis* 136:432–435.
- Dubey JP, Jones JL: 2008, *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol* 38:1257-1278.
- Dubey JP, Lindsay DS, Speer CA: 1998, Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 11:267–299.
- Eliasson M, Olsson A, Palmcrantz E, et al.: 1988, Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. *J Biol Chem* 263: 4323-4327.
- Ferguson DJP, Dubremetz JF: 2007, The ultrastructure of *Toxoplasma gondii*. In: *Toxoplasma gondii—the model apicomplexan: perspectives and methods*, ed. LM Weiss, Kim K, 1st ed., pp. 19–46. Academic Press (Elsevier), London, UK.
- Greiner M, Pfeiffer D, Smith RD: 2000, Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Prev Vet Med* 45:23-41.

- Jacobs L, Lunde MN: 1957, A hemagglutination test for toxoplasmosis. J Parasitol 43:308-314..
- Kijlstra A, Meerburg BG, Mul MF: 2004, Animal-friendly production systems may cause re-emergence of *Toxoplasma gondii*. NJAS 52:119–132.
- Lindsay DS, Dubey JP: 2007, Toxoplasmosis in wild and domestic animals. *In: Toxoplasma gondii—the model apicomplexan: perspectives and methods*, ed. LM Weiss, Kim K, 1st ed., pp. 133–147. Academic Press (Elsevier), London, UK.
- McNemar, Q: 1947, Note on the sampling error of the difference between correlated proportions or percentages. Psychometrika 12:153-157.
- Montoya JG, Liesenfeld O: 2004, Toxoplasmosis. Lancet 363:1965–1976.
- Remington JS, Thulliez P, Montoya JG: 2004, Recent developments for diagnosis of toxoplasmosis. J Clin Microbiol 42:941–945.
- Schaefer JJ, White HA, Schaaf SL, et al.: 2011, Modification of a commercial *Toxoplasma gondii* immunoglobulin G enzyme-linked immunosorbent assay for use in multiple animal species. J Vet Diagn Invest 23:297-301.
- Speer CA, Dubey JP: 1998, Ultrastructure of early stages of infections in mice fed *Toxoplasma gondii* oocysts. Parasitology 116:35–42.
- Surolia A, Pain D, Khan MI: 1982, Protein A: nature's universal anti-antibody. Trends Biochem Sci 7:74–76.
- Werre SR, Jacobson RH, Bowman DD, et al.: 2002, Evaluation of kinetics and single-read enzyme-linked immunoassays for detection of *Toxoplasma gondii* antibodies in sheep. J Vet Diagn Invest 14:225–230.
- Wilson M, McAuley JB: 1991, Laboratory diagnosis of toxoplasmosis. Clin Lab Med 11:923–939.
- Wilson M, Ware DA, Walls KW: 1987, Evaluation of commercial serodiagnostic kits for toxoplasmosis. J Clin Microbiol 25:2262–2265.
- Zhang D, Wang Z, Fang R, et al.: 2010, Use of protein AG in ELISA for serodiagnosis of *Toxoplasma gondii* infection in four species of animals. Clin Vaccine Immunol 17:485–486.

CHAPTER FOUR

***TOXOPLASMA GONDII* SEROPREVALENCE IN NEW YORK STATE WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)**

ABSTRACT

Serum samples collected from 299 white-tailed deer (*Odocoileus virginianus*) that were killed in New York State by hunters in November 2010 were assayed for the presence of anti-*Toxoplasma gondii* IgG antibodies. White-tailed deer are a useful sentinel for risk of human and domestic animal exposure to *Toxoplasma* oocysts and a potential risk for infection to humans and other animals by ingestion of the meat. This herbivorous species shares grazing space with domestic animals raised for meat, and is likely to be exposed by horizontal transmission through oocyst consumption, similar to other grazing species of economic concern. Overall, 42.2% of serum samples in the present study were seropositive on an enzyme-linked immunosorbent assay (ELISA), indicating a true prevalence of 38.5%, with a significantly higher proportion of adult than immature deer testing seropositive. No significant difference in seropositivity was noted between male and female deer, nor was there significant correlation between local human population density and deer seropositivity. These results provide insight on the risk of environmental *Toxoplasma* exposure in New York State and support horizontal transmission through oocyst consumption as the most common mechanism of white-tailed deer infection.

INTRODUCTION

Toxoplasmosis is caused by infection with *Toxoplasma gondii*, and is considered to be one of the most common protozoal infections in the world and the third leading cause of human death from foodborne disease in the United States (Lindsay *et al.*, 2003; Mead *et al.*, 1999). Human infection is common, though it varies significantly across different geographic areas (Dubey, 2010). Exposure can be due to vertical or horizontal transmission, with the latter considered the most common means, by consumption of tissue from an infected intermediate host or by ingestion of oocysts produced by the definitive hosts, domestic and wild felids (Dubey, 2008). Significant morbidity from *Toxoplasma* is uncommon in most infections, with the exception of fetal infection due to exposure of the mother and infection in immunocompromised individuals. Similarly, animal infection is quite common across a broad range of both domestic and wild warm blooded species, with little pathology typically noted. A particular concern is the infection of food animals, both due to potential reproductive effects in the animals and the risk of tissue cysts to consumers in the meat or other tissues (Dubey and Jones, 2008). Toxoplasmosis is of known economic concern among goat and sheep producers and an emerging concern among camelid producers (More *et al.*, 2008).

It can be assumed, barring supplementation with or inadvertent ingestion of animal products, that grazing animals are primarily exposed by either vertical transmission from infected mothers or by consuming oocysts on contaminated feedstuffs. The population density and gregarious nature of deer make them a suitable proxy to characterize risk factors for infection in common with other grazing animals

(Kirchgeßner *et al.*, 2012), and the use of their meat for human and animal consumption makes their infection with *Toxoplasma* a point of interest for public and domestic animal health. The importance of deer as a potential source of infection has been suggested by research noting a higher degree of *Toxoplasma* exposure among consumers of venison than among the general population (Ross *et al.*, 2001; Sacks *et al.*, 1983).

White-tailed deer are the subject of intensive control efforts related to their propensity to cause physical damage to property and as a species contributing to the spread and maintenance of zoonotic and economically significant diseases (DeNicola *et al.*, 2000; Forrester, 1992). Control efforts aimed at reducing *Ixodes scapularis* (black-legged or deer tick) populations in the northeastern US by targeting deer have met with some success, but also have emphasized the difficulties of control of such a large and mobile wildlife population. Additionally, deer live in intimate association with human and domestic animal populations, making some lethal control efforts difficult and often unpalatable to the local residents (Porter *et al.*, 1991). These factors make deer a useful sentinel species for the study of the prevalence of pathogenic agents that pose a risk to humans and other animals, and the ready availability of tissue samples during hunting season provides a unique opportunity to test significant numbers. Previous research has noted *Toxoplasma* seroprevalences in white-tailed deer that ranged from 30% to 64.2% in US state surveys (Dubey, 2010; Dubey *et al.*, 2009; Vanek *et al.*, 1996). A seroprevalence of 60% was detected among deer killed in Pennsylvania, the closest previous study to the present population (Humphreys *et al.*, 1995). There are no reports of clinical toxoplasmosis in white-tailed deer, suggesting that infection is not be

a predisposing factor in being harvested by hunters, though more research on this point is of interest given behavior anomalies observed in other intermediate hosts (Afonso *et al.*, 2012; Gatkowska *et al.*, 2012).

The examination of *Toxoplasma* seroprevalence in the population of hunter-killed New York State deer can provide insight into the dynamics of infection among wildlife and domestic animal populations. Inferences can be made regarding the level of exposure based on sex, age, and the human population density of the town in which the deer were killed. Differences in male vs. female levels of infection may suggest sex-based risk factors promoting exposure. Age based differences in infection level can help to determine how the population of deer have been exposed; e.g. a constant level of infection across age groups would suggest a larger role for vertical exposure, whereas an increasing level of infection with age would suggest horizontal transmission through environmental exposure as a more important route. Finally, the interaction between the local human population density and infection levels in the associated deer may provide insight into the importance of the presence of domestic cats in serving as a route of exposure. Human population will be used as a proxy for cat population, as accurate estimates of the latter are lacking, whereas census based data for the former is available. However, discrepant results between level of cat population density and seroprevalence in deer may be due to management factors, *i.e.*, rural owners may be more likely to allow cats to roam freely outside, leading to more potential oocyst contamination than would be seen with urban, primarily indoor cats. Additionally, a significant level of *Toxoplasma* infection was noted in Pennsylvania bobcats (*Lynx rufus rufus*) which are able to shed oocysts into the environment (Mucker *et al.*, 2006). This

contribution is expected to be minimal compared to that of domestic cats, as the bobcat population in New York State is estimated at only 5,000 (NYS DEC, 2012).

This study describes analysis of 299 white-tailed deer serum samples from animals killed during November 2010 across New York State for antibodies to *Toxoplasma* as an indication of their exposure history. Whole blood was collected from the thoracic cavity of freshly killed deer at deer processing establishments across the state, processed to serum, frozen and stored at the State University of New York's College of Environmental Science and Forestry pending further analysis (Kirschgessner *et al.*, 2012). Serum samples were assayed by an enzyme-linked immunosorbent assay effective for the detection of IgG in a broad range of animal species (Schaefer *et al.*, 2012). Demographic information that had been collected for the deer was contrasted between seropositive and seronegative deer to identify significant risk factors. Results suggest broad characterizations that can distinguish seropositive from seronegative deer and lend themselves to risk analysis for meat producers and consumers of venison.

MATERIALS AND METHODS

Sample Processing

Blood samples were collected from the thoracic, nasal or oral cavities of hunter-harvested white-tailed deer at private deer processing facilities and New York State Department of Environmental Conservation (NYSDEC) deer check stations across New York State. Collections were performed November 20-21, 2010, the first weekend of

the 2010-2011 regular firearm season in the majority of the state. Blood was transferred to 10-mL glass serum tubes and stored at 4°C prior to centrifugation at 1300 g for 10 minutes. Supernatant (serum) was collected and frozen at -80°C pending further analysis (Kirchgessner *et al.*, 2012). A total of 299 serum samples, 100 from females and 199 from males, were evaluated as described below.

ELISA

Serum samples were tested for the presence of anti-*Toxoplasma* IgG using the technique described in Schaefer *et al.*, 2012. The Wampole Laboratories' *Toxoplasma gondii* IgG ELISA II kit provided antigen coated wells were filled with the serum of interest, allowed to incubate and washed with kit supplied wash buffer. Protein A/G conjugate diluted to 1:40,000 with PBS was used for detection of antigen-antibody bonds instead of the kit-supplied anti-human IgG conjugate. Following incubation and a second wash step, tetramethylbenzidine (TMB) was added as an indicator of the presence of conjugate and color intensity was measured on a microplate reader at a wavelength of 450 µm. Optical density ratios were calculated using kit supplied standards and used to determine serological status.

Data Analysis

Samples were categorized as “positive” vs. “negative” based on the results of the ELISA assay. Results in the equivocal range were noted for 31 of the samples, which were excluded from further analysis. Positive samples were considered cases and negative samples were considered controls for purposes of risk factor interpretation. A logistic regression was performed using JMP9 software with variables of age (less than

one year old vs. greater than one year old), sex and human population density in the town in which each deer was killed with output of serological status. Significant factors contributing to serological status were identified and further analyzed by determining odds ratio with 95% confidence intervals. ArcGIS10 was used to produce a New York State map indicating towns from which seropositive deer were identified and those in which none of the deer sampled for this investigation were positive for *Toxoplasma* antibody.

RESULTS

Samples that produced optical density ratio values in the equivocal range were excluded from analysis, leaving 113 samples that were determined to be positive and 155 that were negative for *Toxoplasma* exposure, giving an apparent prevalence of 42%. The ELISA test had previously been demonstrated to have a sensitivity and specificity of 92% and 89%, respectively, using agglutination testing as a gold standard (Schaefer *et al.*, 2012). Using calculations for true prevalence based on test performance, our testing indicates a prevalence of 38.5% (95% confidence interval of 31.2 - 45.8%) for this population of deer (Rogan and Gladen, 1978).

Logistic regression analysis was conducted using JMP9, with age, sex and town population density as predictors and serological status as the outcome. Age of > 1 year was determined to be a significant factor in increasing risk of seropositivity ($p=0.0013$), whereas the effects of sex and local human population density were found to be insignificant ($p=0.4831$ and 0.1285 , respectively). Seropositivity values were 20.6% for

the younger group vs. 46.8% for the older group. The odds ratio for seropositive deer that were greater than a year old to those that were less than a year old was calculated to be 3.39 (95% confidence interval of 1.42-8.12). When distinguished by sex, 38.9% of females were seropositive on the ELISA, vs. 43.8% of the males. Human population density was analyzed as a continuous variable, with no significant trend in relation to seropositivity detected.

Locations from which deer were collected were noted below on a map of upstate New York created using ARCGIS10, with diamonds indicating the towns from which deer were collected. Black diamonds indicate those towns from which at least one deer was found to be seropositive for *Toxoplasma*, whereas white diamonds indicate those in which none of the deer killed were found to be seropositive. Shading increase with increasing human population density by zip code as noted (Figure 4.1).

DISCUSSION

The widespread exposure of deer to *Toxoplasma* in New York State is indicated by a seroprevalence of 38.5% in this group of deer killed by hunters in November 2010. This level is consistent with prevalences described from other states, but lower than that 60% noted in a Pennsylvania study from 1995 (Dubey, 2010; Humphreys *et al.*, 1995). Older deer were noted to have a significantly higher risk of being seropositive, whereas no significant difference was noted between male and female levels of exposure. The local human population density did not appear to be a risk factor for infection. This is consistent with infection of the deer by horizontal transmission, with the risk of having

Legend

USA Population by Zip Code

Population 2010

Over 36,000

28001 - 36000

18001 - 28000

9001 - 18000

3001 - 9000

1001 - 3000

1 - 1000

Zero or No Data

◇ NYDeerNeg.csv Events

◆ NYDeerToxoPos.csv Events

80

Kilometers

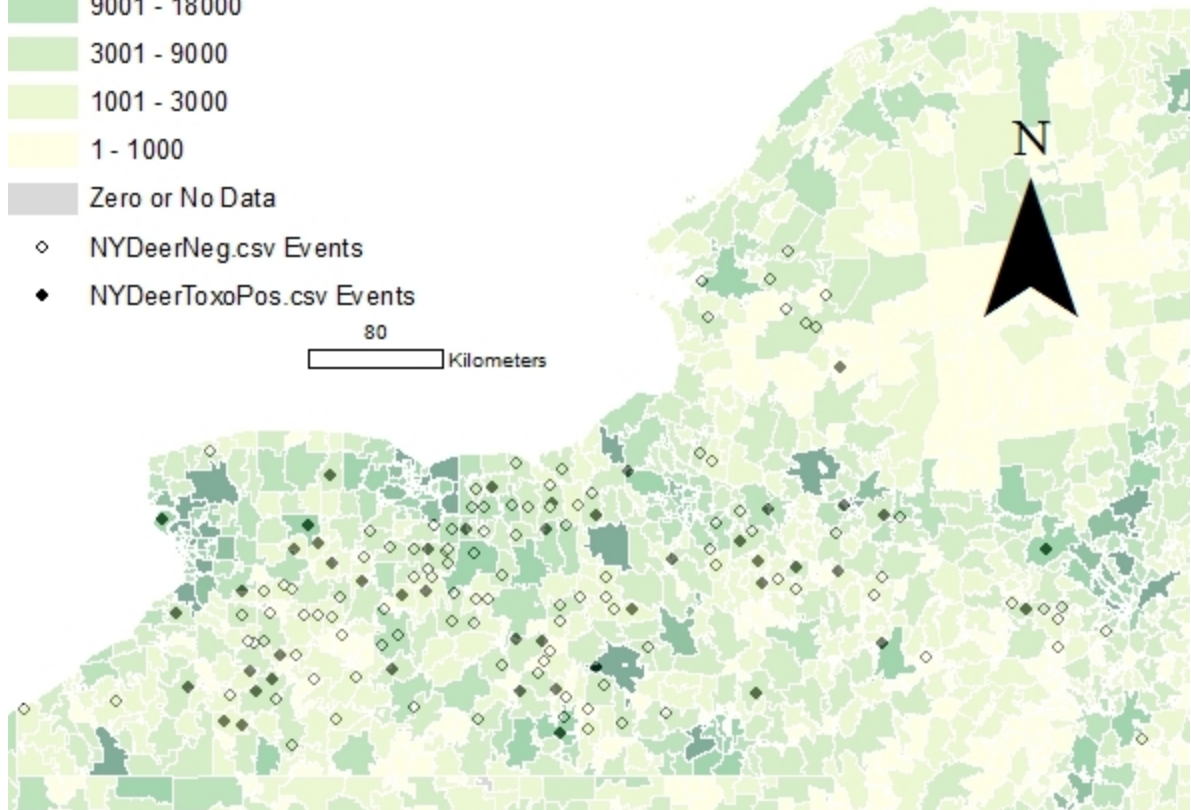


Figure 4.1. Localities from which seropositive deer were collected (◆) and from which only seronegative deer were collected (◇), with background shading indicating human population density. Produced with ARCGIS10.

ingested oocysts increasing with increasing age. A map of upstate New York indicating collection sites and the presence or absence of seropositive samples was created. It should be noted that these were convenience samples with sites and the numbers of samples from each site not being standardized. No conclusions regarding these specific locations and the presence or absence of *Toxoplasma* oocysts in the environment should be derived from these results beyond noting the widespread nature of seropositive sites.

The relation to human population analysis was performed based on the town in which the deer was killed. It has been found that average deer ranges are 2.8 – 4.9 km² in yearling male white-tailed deer, with female deer known to have a more limited range (McCoy *et al.*, 2005). A New York study conducted in the Adirondacks noted an average range for white-tailed deer of 2.25 km² in the summer and 1.35 km² in the winter (Tierson *et al.*, 1985). The towns from which these deer were collected are generally greater than 100 km² in area, making it likely that most *Toxoplasma* exposures would have occurred in the town of collection, but overlap with neighboring towns is certain to be seen. When human population density was analyzed as a continuous variable, no significant prediction of seropositivity was noted. This group of samples was highly skewed for analysis since most of the deer were collected from rural towns with a median human population density of 25 residents/km². In one measure, the United States Census considers urban areas as those with at least 386 residents/km². Future sample collections including more populated Hudson River Valley suburban townships that allow hunting will allow comparison of *Toxoplasma* prevalence in deer from towns with a wider range of population densities.

The potential relationship between human population density and *Toxoplasma* seropositivity in deer may be due to a greater domestic feline population density and therefore greater environmental oocyst contamination. Since this is the expected route of exposure for deer it needs to be investigated further. Deer feeding behavior in areas of greater human population density may be expected to include grazing closer to human habitations, areas in which owned cats are more likely to defecate. This scenario is based on domestic cats being the singular source of *Toxoplasma* oocysts. Bobcats, the only other expected feline source of oocysts in the region, comprise a fraction of the domestic cat population, as noted above. Conversely, rural areas might be expected to harbor more cats that spend time outdoors and participate in rodent consumption, thereby being more likely to be infected with *Toxoplasma*. Interestingly, a study in Ohio noted greater exposure in deer from areas of lower human population density (Crist *et al.*, 1999), potentially supporting the latter scenario. Further investigation including deer from more diverse areas in the state will help to clarify this question, and thereby provide a more accurate estimate of factors that contribute to *Toxoplasma* infection in deer and their role in human exposure.

REFERENCES

- Afonso C, VB Paixao, RM Costa. 2012. Chronic *Toxoplasma* infection modifies the structure and the risk of host behavior. PLoS ONE 7: e32489.
- Brillhart, DB, LB Fox, JP Dubey, SJ Upton. 1994. Seroprevalence of *Toxoplasma gondii* in wild mammals in Kansas. J. Helminthol. Soc. Wash. 61: 117-121.
- Crist SC, RL Stewart, JP Rinehart and GR Needham. 1999. Surveillance for *Toxoplasma gondii* in the white-tailed deer (*Odocoileus virginianus*) in Ohio. Ohio J Sci 99: 34-37
- DeNicola AJ, DC VerCauteren, PD Curtis, SE Hygnstrom. 2000. Managing white-tailed deer in suburban environments: a technical guide. Cornell Cooperative Extension. Ithaca, NY.
- Diefenbach DR, ES Long, CS Rosenberry, BD Wallingford, DR Smith. 2008. Modeling distribution of dispersal distances in male white-tailed deer. J Wildl Manage 72: 1296-1303
- Dubey JP. 2008. The history of *Toxoplasma gondii* – the first 100 years. J Eukary Microbiol 55: 467-475.
- Dubey JP. 2010. Toxoplasmosis of Animals and Humans, 2nd Edition. CRC Press, Boca Raton, FL.
- Dubey, JP, DH Graham, RW de Young, E. Dahl, ML Eberhard, EK Nace, K Won, H Bishop, G. Punkosdy, C Sreekumar, MCB Vianna, SK Shen, OCH Kwok, JA Sumners, S Demarais, JG Humphreys and T Lehmann. 2004 Molecular and biologic characteristics of *Toxoplasma gondii* isolates from wildlife in the United States. J Parasitol. 90: 67-71
- Dubey, JP, MC Jenkins, OCH Kwok et al., 2009. Seroprevalences of *Neospora caninum* and *Toxoplasma gondii* antibodies in white-tailed deer (*Odocoileus virginianus*) from Iowa and Minnesota using four serologic tests. Vet Parasitol. 161: 330-334.
- Dubey JP, JL Jones. 2008. *Toxoplasma gondii* infection in humans and animals in the United States. Int J Parasitol 38: 1257-1278.
- Forrester DJ. 1992. Parasites and diseases of wild mammals in Florida. University Press of Florida. Gainesville, FL.
- Gatkowska J, M Wieczorek, B Dziadek, K Dzitko, H Dlugonska. 2012. Behavioral changes in mice caused by *Toxoplasma gondii* invasion of brain. Parasitol Res 111: 53-58.

- Humphreys, JG, RL Stewart and JP Dubey. 1995. Prevalence of *Toxoplasma gondii* antibodies in sera of hunter-killed white-tailed deer in Pennsylvania. *Am J Vet Res* 56: 172-173.
- Kirchgessner, MS, EJ Dubovi and CM Whipps. 2012. Seroepidemiology of *Coxiella burnetii* in wild white-tailed deer (*Odocoileus virginianus*) in New York, United States. *Vector Borne Zoonotic Dis* 12: 942-947.
- Lindsay, DS, BL Blagburn, JP Dubey and WH Mason. 1991. Prevalence and isolation of *Toxoplasma gondii* from white-tailed deer in Alabama. *J. Parasitol.* 77: 62-64.
- Lindsay DS, LM Weiss, Y Suzuki. 2003. Toxoplasmosis in North American Parasitic Zoonoses. *World Class Parasites Volume 6*. SJ Black, JR Seed, eds. 129-150
- McCoy JE, DG Hewitt and FC Bryant. 2005. Dispersal by yearling male white-tailed deer and implications for management. *J Wildl Manage* 69: 366-376.
- Mead PS, L Slutsker, V Dietz, LF McCaig, JS Bresee, C Shapiro, PM Griffin, RV Tauxe. 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5: 607-624.
- More G, L Pardini, W Basso, R Marin, D Bacigalupe, G Auad, L Venturini, MC Venturini. 2008. Seroprevalence of *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* sp. in llamas (*Lama glama*) from Jujuy, Argentina. *Vet Parasitol* 155: 158-160.
- Mucker EM, JP Dubey, MJ Lovallo, JG Humphreys. 2006. Seroprevalence of antibodies to *Toxoplasma gondii* in the Pennsylvania bobcat (*Lynx rufus rufus*). *J Wildl Dis* 42: 188-191.
- New York State Department of Environmental Conservation. 2012. Management plan for bobcat in New York State 2012-2017.
- Nixon CM, LP Hansen, PA Brewer, JE Chelsvig, JB Sullivan, TL Esker, R Koerkenmeier, DR Etter, J Cline, JA Thomas. 1994. Behavior, dispersal and survival of male white-tailed deer in Illinois. *Illinois Natural History Survey Biological Notes* 139: 30 pp.
- Porter WF, NE Mathews, HB Underwood, RW Sage Jr., DF Behrend. 1991. Social organization in deer: implications for localized management. *Environ Manage* 15: 809-814.
- Rogan WJ, B Gladen. 1978. Estimating prevalence from the results of a screening test. *Am J Epidemiol* 107: 71-76.

- Ross RD, LA Stec, JC Werner, MS Blumenkranz, L Glazer, GA Williams. 2001. Presumed acquired ocular toxoplasmosis in deer hunters. *Retina* 21: 226-229.
- Sacks JJ, DG Delgado, HO Lobel, RL Parker. 1983. Toxoplasmosis infection associated with eating undercooked venison. *Am J Epidemiol* 118: 832-838.
- Tierson WC, GF Mattfeld, RW Sage Jr., DF Behrend. 1985. Seasonal movements and home ranges of white-tailed deer in the Adirondacks. *J Wildl. Manage* 49: 760-769.
- Vanek, JA, JP Dubey, P. Thulliez, MR Riggs and BE Stromberg. 1996. Prevalence of *Toxoplasma gondii* antibodies in hunter-killed white-tailed deer (*Odocoileus virginianus*) in four regions of Minnesota. *J Parasitol.* 82: 41-44.

CHAPTER FIVE

PCR DEVELOPMENT AND DETECTION OF *TOXOPLASMA GONDII* DNA IN INFECTED DOGS (*CANIS FAMILIARIS*) AND SHEEP (*OVIS ARIES*)

ABSTRACT

The antemortem diagnosis of *Toxoplasma* infection in animals is typically accomplished by detection of IgG or IgM antibody in serological samples. While demonstrating a history of exposure to *Toxoplasma gondii*, serological testing cannot definitively determine the stage or severity of the infection with precision. The ability to speciate *Toxoplasma*-like oocysts and tissue cysts will assist both diagnostic and etiological investigation. The advent of molecular methods allows both more specific description of protozoal infections, as well as the ability to localize the infection in distinct anatomical locations. Following determination of an optimal primer set for detection, a real time quantitative polymerase chain reaction was developed and tested against both positive control samples derived from *Toxoplasma* tachyzoite culture and canine and ovine serum samples that were determined to be positive for antibody by agglutination testing. The limit of detection was determined to be four copies using cloned DNA of known concentration. Neither *Toxoplasma* positive nor negative serum from goats and dogs produced positive results on the PCR assay, suggesting serum is an inappropriate tissue for diagnostic testing using PCR.

INTRODUCTION

Serological detection has been the primary method for the diagnosis of *Toxoplasma* infection in both humans and animals. Direct detection of the parasite is complicated in the definitive feline host by the transient appearance of oocysts in feces and in intermediate hosts by the difficulty in predicting the precise location of tissue cysts (Dabritz and Conrad, 2010; Dabritz *et al.*, 2007; Dubey, 1995; Dubey, 2001; Dubey *et al.*, 1977). While serology provides evidence of past exposure, a single sample does not allow the characterization of an acute versus a chronic infection, nor the severity of the infection. The direct detection of DNA by polymerase chain reaction seems to be a promising method in that a positive result establishes the presence of the organism in the tested tissue. However, the nature of the organism's complex life cycle poses a challenge in translating a successful PCR assay into a useful clinical test.

Advent of PCR for *Toxoplasma*

There are two commonly used targets for the detection of genomic DNA by either traditional or real time PCR, the *B1* gene and AF146527 (529 base pair repeat element). The *B1* gene was initially detected as a PCR target with 35 repeats in the *Toxoplasma* genome by Burg *et al.* (1989), who determined a limit of detection of one organism in cell lysate and 10 organisms in a human leukocyte preparation by traditional PCR, with no evidence of detection of closely related pathogens. The *B1* gene has become a standard target for PCR detection of *Toxoplasma* in clinical samples and, significantly for veterinary diagnostics, does not occur in *Neospora*

caninum (Chabbert *et al.*, 2004; Schatzberg *et al.*, 2003). More recently, a 529 base pair target (AF146527) that is repeated 200-300 fold in the *Toxoplasma* genome has been described and utilized as a target for PCR detection (Homan *et al.*, 2000). No statistically significant difference in sensitivity or specificity between the *B1* gene and AF146527 targets were noted in some investigations (Filisetti *et al.*, 2003; Remington *et al.*, 2004), whereas other studies noted that assays for 529 were more sensitive for a variety of fluid and tissue samples (Calderaro *et al.*, 2006; Cassaing *et al.*, 2006; Dubey, 2010; Homan *et al.*, 2000; Reischl *et al.*, 2003). Investigation of sequence variation across *Toxoplasma* strains from human infections noted that the AF146527 element is absent in 4.8% of cases, which may lead to false negative determinations even if the organism is present in the tissue being evaluated (Wahab *et al.*, 2010).

Current utility of PCR

Both methods are used for detection of *Toxoplasma* in diverse tissues in human and veterinary diagnostic investigation. Samples commonly evaluated from suspected human cases include: amniotic fluid, placenta, brain, blood, cerebrospinal fluid, urine, vitreous, aqueous, bronchoalveolar lavage, pleural and peritoneal fluids (Remington *et al.*, 2004). Veterinary diagnostic laboratories offer evaluation of blood, feces, CSF, brain and other tissues, with fecal examination used to distinguish *Toxoplasma* oocysts from morphologically similar *Hammondia hammondi* in feline feces (Schaes *et al.*, 2008). Knowledge of the parasite's life cycle is critical to increase the utility of PCR testing, e.g. blood stages are transient and expected early in infection, and detection of the parasite in tissue relies on the presence of a tissue cyst in the aliquot being tested.

Even with a good sampling strategy, molecular detection must be considered a method of unproven sensitivity but good specificity, where the predictive value of a positive result is much higher than that of a negative result (Remington *et al.*, 2004). This makes molecular detection a reasonable adjunct to serological investigation, in which the opposite is true regarding positive and negative predictive values. The establishment of a reliable real time PCR protocol for detection of *Toxoplasma* DNA will allow definitive diagnosis of the presence and stage of infection when serological testing produces equivocal results. This study describes a comparison of protocols for *Toxoplasma* detection by traditional PCR and the development of a real time PCR assay to test on serum samples from seropositive and seronegative dogs and sheep.

MATERIALS AND METHODS

Cell Culture

Cultured tachyzoites were acquired from the laboratory of Dr. Eric Denkers (Cornell University, Ithaca, NY). Tachyzoites of Type I *Toxoplasma* strain RH had been maintained by bi-weekly passage on human foreskin fibroblast monolayers (American Type Tissue Collection) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% Bovine Growth Serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Two 100 µl aliquots of culture media containing approx. 5,000,000 tachyzoites each were subjected to DNA extraction.

Sample Collection and Processing

Blood samples were collected in clot tubes from animals by referring veterinarians to allow serum separation and collection. They were submitted to the Animal Health Diagnostic Center at Cornell University (Ithaca, New York) from 2002 to 2007 for the detection of *Toxoplasma* exposure by indirect hemagglutination. Nine serum samples were used for this study, including four canine samples (three seropositive and one seronegative) and five ovine samples (four seropositive and one seronegative).

DNA Extraction

DNA was extracted from both the cultured tachyzoites and 200 µl aliquots of sera using the QIAamp DNA Mini Kit's protocol. The ratio of 260 nm to 280 nm and concentration of DNA was tested using a nanodrop spectrophotometer (Table 5.1).

Primer Development

Two primer sets were chosen based on previously published work using the *B1* gene and 529 targets, the former amplifying a 94-bp internal fragment and the latter a 98-bp internal fragment (Edvinsson *et al.*, 2006). The 529-bp repeat element exists in the genome in 200-300 copies, whereas the *B1* gene occurs in 35 copies. This leads to the prediction that an assay targeting the 529-bp repeat element would be more sensitive than one targeting the *B1* gene. Experimental results attempting to verify this prediction have been mixed, with some detecting greater sensitivity using the 529 assay

and others detecting no significant difference (Cassaing *et al.*, 2006; Filisetti *et al.*, 2003). The *B1* gene and 529-bp repeat element published primers were used and results were compared for positive controls in the presence and absence of serum and extracted sera on traditional PCR assays. Additionally, two new primers targeting the 529-bp repeat element were developed and similarly evaluated. The primers that produced significant bands at the predicted size without the presence of non-target bands (in this case the *B1* gene primers) were then used in a Sybr-green real-time PCR assay to detect the limit of their sensitivity using a cloned amplicon.

Novel primer sets for the detection of *Toxoplasma* DNA by targeting the 529-bp repeat region were developed using the published sequence designated AF146527 on the National Center for Biotechnology Information's (NCBI's) website. Primers were chosen based on the design recommendations described in Bio Rad Laboratories website (www3.bio-rad.com): guanine-cytosine (GC) content of 50-60%, melting temperature between 50-65° C, lack of secondary structure, no G or C repeats greater than 3 bases and Gs and Cs on the end of the primers. One forward primer and two reverse primers were identified based on these criteria, with the aid of Dr. Michael Zuker's DNA mfold server to analyze secondary structures (www.bioinfo.rpi.edu/applications/mfold/). The predicted amplicon sizes were 135 bp and 116 bp (Table 2).

Table 5.1. DNA quantitation in extracted *Toxoplasma* culture and serum samples.

Sample	260/280	Concentration (ng/μl)
Tachyzoites #1	2.35	31.6
Tachyzoites #2	2.26	31.1
Sample #1 (+ canine)	1.15	51.5
Sample #2 (+ canine)	1.20	9.7
Sample #3 (+ canine)	1.25	19.6
Sample #4 (- canine)	1.66	6.7
Sample #5 (+ ovine)	1.33	9.8
Sample #6 (+ ovine)	1.08	7.0
Sample #7 (- ovine)	1.34	6.5
Sample #8 (+ ovine)	1.23	45.6
Sample #9 (+ ovine)	1.00	16.2
Distilled water	1.64	2.2

Table 5.2. Primers used to amplify indicated target.

TARGET	Size(bp)	Primer 1	Primer 2
B1 gene	98	CGT CCG TCG TAA TAT CAG	GAC TTC ATG GGA CGA TAT G
529 element	94	CAC AGA AGG GAC AGA AGT	TCG CCT TCA TCT ACA GTC
529(js1)	135	CTT GGA GCC ACA GAA GGG	CTT CCC AAC CAC GCC ACC
529(js2)	116	CTT GGA GCC ACA GAA GGG	CCT CAT CCT CAC CCT CGC

Traditional PCR

The four primer sets were tested using positive control DNA extracted from cultured tachyzoites. All four assays produced positive results in duplicate at the predicted size as indicated by Figure 5.1. Published primers 529 were subjectively noted to have produced less significant bands, while primers 529js2 produced non-target banding, which can complicate quantitative interpretation if utilized on a real-time platform. Therefore primers for B1 gene and 529js1 were used for evaluation of clinical samples, with no amplification of seropositive nor seronegative dog and goat samples detected (Figure 5.2). Non-target banding was noted in 529js1, leading to the choice of B1 gene primers for further development.

Cloning

B1 gene primers were used for real-time PCR analysis of positive controls and samples following traditional PCR analysis. The *Escherichia coli* (*E. coli*) plasmid cloning vector pUC19 was prepared for cloning using the Qiagen Plasmid Purification Kit (Midi). After establishing a significant concentration of DNA present in the resulting solution (555 ng/μl), plasmid DNA was cut and linearized by digestion with restriction enzymes EcoR1 and Pst1. Agarose gel electrophoresis allowed separation of the larger (2646 bp) from the smaller (40 bp) fragment and the latter was collected by physical excision and the use of a gel extraction kit (QIA Quick PCR Purification). B1 gene primers were modified by the addition of EcoR1 sequence to the forward and Pst1 sequence to the reverse, and used to produce an abundance of amplicon by the same technique. The plasmid and B1 gene amplicon were ligated by use of the Fast-Link

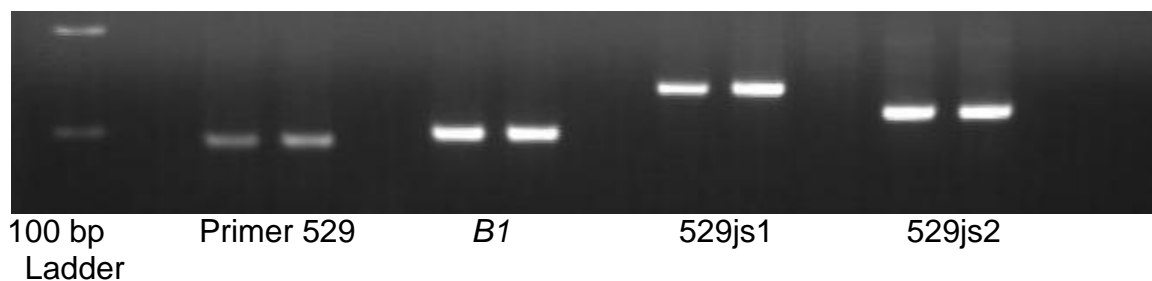


Figure 5.1. Results of traditional PCR testing of 31.1 ng positive control using (from left to right) primers for 529 repeat element, *B1* gene, and newly developed primers 529js1 and 529js2.



Figure 5.2. *B1* gene and 529js1 evaluation of extracted canine and caprine sera: (left to right) 100 bp ladder, *B1* gene assay (3 ng positive control, negative control, + dog, - dog, - goat, + goat, extracted H2O) 529 repeat sequence assay (same order of samples).

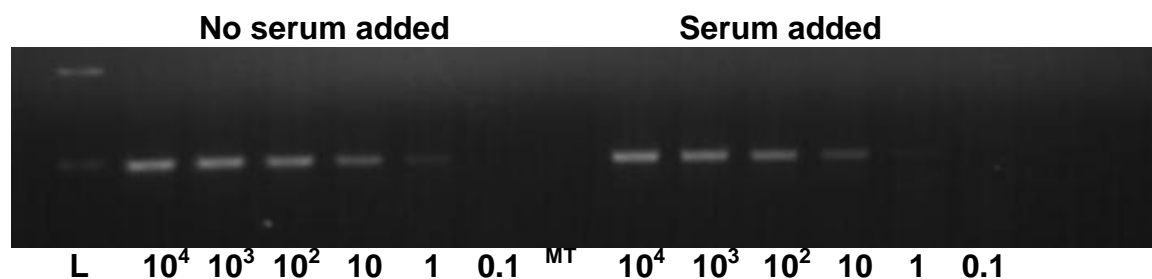


Figure 5.3. Lack of inhibition of negative serum (canine) – left dilution series of positive control without serum, right with serum (approximate number of organisms noted)

DNA Ligation kit (Epicentre Biotechnologies) then added to lysogeny broth (LB) containing DH5α *E. coli*. Following plating on LB, appropriate colonies were removed and transferred to ampicillin plates, then broth to select for plasmid containing bacteria and to allow further growth. QIA preparation mini kits were used to extract DNA from the recombinant bacteria and linearization using restriction enzyme *ScaI* was performed. The QIA Quick PCR Purification Kit was then used to isolate the linearized plasmid including the insert DNA in two aliquots. The resultant DNA concentration and 260/280 ratios were 68.1 and 79.0 ng/μl and 1.91 and 1.89, respectively. This represents a relatively pure sample of target DNA when compared to the positive control extracted from cultured cells, and allows a more accurate evaluation of test sensitivity.

Real Time PCR

The molecular weight of each plasmid containing the insert was estimated at approximately .003 femtograms. This information was used to create a dilution series from 2.27×10^9 to 0.0227 particles/μl. Initially a traditional PCR with B1 gene primers was used, with 5 μl of template per reaction, to give a dilution series from 142,000 particles to 0.142 particles to be evaluated. Results seemed to indicate a very good sensitivity, however a light band at the same target size was present in the negative control well on multiple observations, so no definitive sensitivity was established by this technique. The plasmid with insert DNA was reevaluated using real time PCR using a dilution series of 40,700 to 0.407 particles per reaction on a Sybrgreen platform.

RESULTS

Traditional PCR testing of a positive control with all four primer sets (B1 gene, 529 repeat element, and the two newly developed 529 primers) all revealed strong positives at the appropriate size (Figure 5.1). However, all three primers based on the 529 repeat element also produced multiple light non-target bands, and were therefore less suitable for Sybr-green real time PCR development due to potential issues with specificity. Testing of extracted sera from seropositive and seronegative dogs and goats did not reveal the presence of *Toxoplasma* DNA at the predicted size by either B1 gene or 529 repeat element assays (Figure 5.2). To determine if serum can cause inhibition of PCR, leading to false negative results, two dilution series from approximately 10,000 to approximately 0.1 organisms were assayed (Figure 5.3). No significant inhibition was noted, as amplification was noted to a similar level (approximately one organism) regardless of the inclusion or exclusion of negative canine serum.

A real time PCR assay was developed as described and three identical dilution series of plasmids with insert were run in duplicate with 0.407 to 40,700 particles per reaction. Negative and positive controls were included and amplified appropriately, notably negative controls did not indicate any significant DNA contamination (Figure 5.4). The concordance between the cycle threshold (Ct) values for each dilution predictably becomes weaker as the number of particles decreases, which is most notable at 0.4 and 4 particles (Figure 5.5). The melt curve did not indicate the

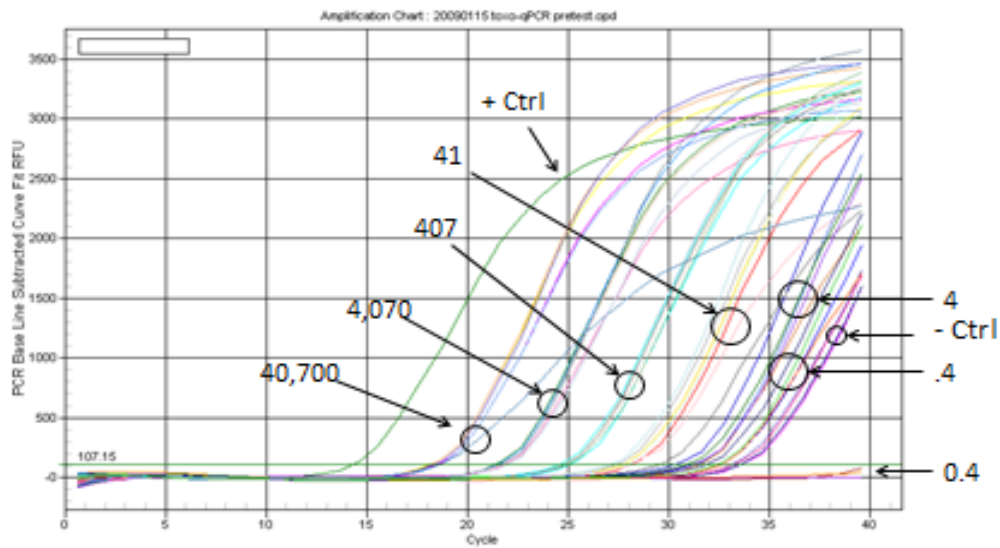


Figure 5.4. Amplification plot with particles per reaction noted

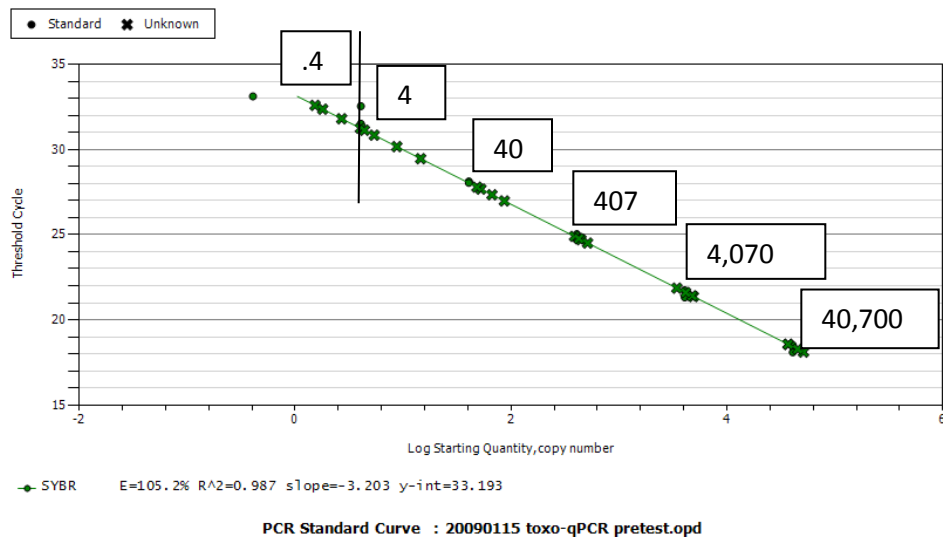


Figure 5.5: Standard curve for dilution series

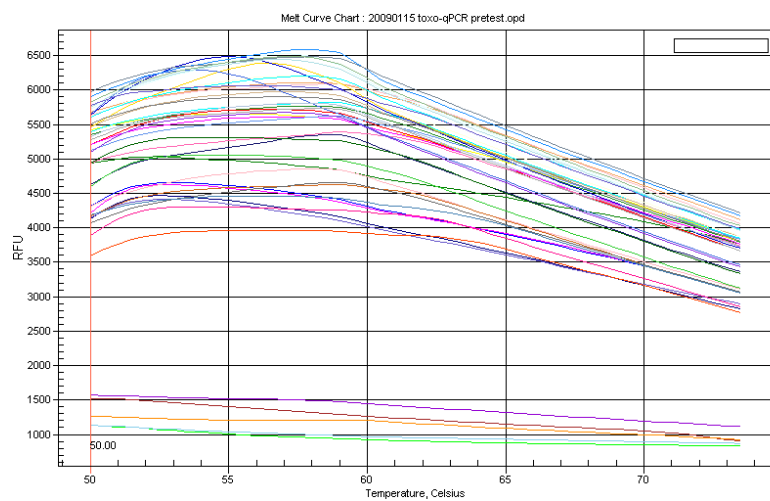


Figure 5.6: Melt curve

amplification of non-target DNA from samples or controls in any significant amount (Figure 5.6).

DISCUSSION

Though the sensitivity of detection of the *Toxoplasma B1* gene versus 529 bp sequence was not directly compared in this study, *B1* gene detection on this real-time platform was demonstrated to be sufficiently sensitive for use in diagnostic investigation. A recent reevaluation of copy numbers of both the *B1* gene and the 529 b.p. sequence determined that the number of repeats of both had been overestimated in previous studies. This is likely due to earlier reliance on blotting as an adjunct to conventional PCR, though the 529 sequence was still noted to occur in greater numbers in the genome than the *B1* gene (Costa and Bretagne, 2012). The lack of detection of parasite DNA from serum samples is not surprising due to the nature of the parasite's life cycle, in which DNA is only present in the intermediate host's vascular system early in infection, demonstrated by detection in acute infection of 48.6% and in chronic infection of 3.6%. Chronicity was characterized by the presence or absence of anti-*Toxoplasma* IgM antibodies (Kompalic-Cristo *et al.*, 2007). In a study examining heart tissue from cattle, *Toxoplasma* DNA was detected in a small number of seronegative animals, with no seropositive animals testing positive (Opsteegh *et al.*, 2011). An investigation using quantitative real-time PCR for detection of the 529 bp element in various tissues from experimentally infected goats determined that the highest concentration of organism was found in lung tissue at 30 and 90 days post inoculation. Concentration of organism in the liver and dorsal muscle tissues increased with time,

whereas that in heart tissue decreased (Jurankova *et al.*, 2012). Of these, liver tissue is the most direct to sample antemortem, though sampling any of the organs has the potential of serious complications.

Further investigation into the utility of PCR for detection of *Toxoplasma* DNA and standardization of a protocol in the veterinary diagnostic setting is warranted. These preliminary findings do not support the use of PCR in serologic diagnosis, but more study is needed. The real time PCR results are promising for detection and predictable quantitation of parasite DNA based on Ct values for ≥ 40 organisms, though the significance of the latter information needs further investigation. PCR has a more significant role in medical diagnostic investigation, though it has not replaced serological tests as the preferred first line method (Remington *et al.*, 2004). The life cycle of *Toxoplasma*, especially the transitory nature of its presence in the vascular system prior to tissue invasion complicates the use of PCR for diagnosis. The method does have utility in distinguishing *Toxoplasma* oocysts from similar appearing oocysts when found by microscopy in feline feces. In intermediate hosts where specific tissue invasion is suspected (e.g. ocular, neurological, fetal) due to the presence of pathognomonic clinical signs, PCR may be the most direct method to definitively establish etiology. Though commercial kits for PCR detection of *Toxoplasma* in tissue are available, comparison to the sensitivity of laboratory developed protocols seems to be unfavorable, possibly linked to the presence of PCR inhibitors in the samples (Morelle *et al.*, 2012). The present study supports the feasibility of using PCR as a diagnostic method in veterinary investigation, while emphasizing the importance of acquiring appropriate tissue for the results to be clinically significant.

REFERENCES

- Burg JL, CM Grover, P Pouletty, JC Boothroyd. 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. J Clin Microbiol 27: 1787-1792.
- Calderaro A, G Piccolo, C Gorrini, S Peruzzi, L Zerbini, S Bommezzadri, G Dettori, C Chezzi. 2006. Comparison between two real-time PCR assays and a nested-PCR for the detection of *Toxoplasma gondii*. Acta Biomed 77: 75-80
- Cassaing S, Bessieres MH, Berry A, Berrebi, A, Fabre R, Magnaval JF: 2006, Comparison between two amplification sets for molecular diagnosis of toxoplasmosis by real-time PCR. J Clin Microbiol 44: 720-724.
- Chabbert E, L Lachaud, L Crobu, P Bastien. 2004. Comparison of two widely used PCR primer systems for detection of *Toxoplasma* in amniotic fluid, blood, and tissues. J Clin Microbiol 42: 1719-1722.
- Costa J-M, S Bretagne. 2012. Variation of *B1* gene and AF146527 repeat element copy numbers according to *Toxoplasma gondii* strains assessed using real-time quantitative PCR. J Clin Microbiol 50: 1452-1454
- Dabritz HA, Conrad PA: 2010, Cats and *Toxoplasma*: implications for public health. Zoonoses Public Health 57:34–52.
- Dabritz HA, Miller MA, Atwill ER, et al.: 2007, Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. J Am Vet Med Assoc 231:1676–1684.
- Dubey JP: 1995, Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J Parasitol 81:410–415.
- Dubey JP: 2001, Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. J Parasitol 87:215–219.
- Dubey JP. 2010. Toxoplasmosis of Animals and Human, 2nd edition. CRC Press. Boca Raton, FL
- Dubey JP, Christie E, Pappas PW: 1977, Characterization of *Toxoplasma gondii* from the feces of naturally infected cats. J Infect Dis 136:432–435.
- Edvinsson B, Lappalainen M, Evengard B: 2006, Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. Clin Microbiol Infect 12: 131-136.
- Filisetti D, Gorcii M, Pernot-Marino E, Villard O, Candolfi, E: 2003, Diagnosis of congenital toxoplasmosis: comparison of targets for detection of *Toxoplasma gondii* by PCR. J Clin Microbiol 41:4826-4828.

- Homan, WL, M Vercammen, J de Braekeleer, H Verschueren. 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol*. 30: 67-95
- Jurankova J, M Opsteegh, H Neumayerova, K Kovarcik, A Frencova, V Balaz, J Volf, B Koudela. 2012. Quantification of *Toxoplasma gondii* in tissue samples of experimentally infected goats by magnetic capture and real-time PCR. *Vet Parasitol*. <http://dx.doi.org/10.1016/j.vetpar.2012.11.016>.
- Kompalic-Cristo A, C Frotta, M Suarez-Mutis, O Fernandes, C Britto. 2007. Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of *Toxoplasma gondii* in human peripheral blood. *Parasitol Res* 101: 619-625.
- Morelle C, E Varlet-Marie, M-P Brenier-Pinchart, S Cassaing, H Pelloux, P Bastien, Y Sterkers. 2012. Comparative assessment of a commercial kit and two laboratory-developed PCR assays for molecular diagnosis of congenital toxoplasmosis. *J Clin Microbiol* 50: 3977.
- Opsteegh M, P Teunis, L Zuchner, A Koets, M Langelaar, J van der Giessen. 2011. Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. *Int J Parasitol* 41: 343-354.
- Reischl U, S Bretagne, D Kruger, P Ernault, J-M Costa. 2003. Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 3: 7.
- Remington JS, Thulliez P, Montoya JG: 2004, Recent developments for diagnosis of toxoplasmosis. *J Clin Microbiol* 42:941–945.
- Schares GD, DC Herrmann, A Beckert, S Schares, M Hosseinienejad, N Pantchev, M Globokar Vrhovec, FJ Conraths. 2008. Characterization of a repetitive DNA fragment in *Hammondia hammondi* and its utility for the specific differentiation of *H. Hammondi* from *Toxoplasma gondii* by PCR. *Mol Cell Probes* 22: 244-251.
- Schatzberg SJ, NJ Haley, SC Barr, A deLahunta, N Olby, K Munana, NJH Sharp. 2003. Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. *AJVR* 64: 1507-1513
- Wahab T, B Edvinsson, D Palm, J Lindh. 2010. Comparison of the AF146527 and B1 repeated elements, two real-time PCR targets used for detection of *Toxoplasma gondii*. *J Clin Microbiol* 48: 591-592.

CHAPTER SIX

CONCLUSION

The development of an assay that can detect the presence of anti-*Toxoplasma* IgG antibody in serum samples from a broad range of animal species benefits veterinary and public health workers, both in identification of individual cases and characterization of the frequency of infection among different animal reservoirs. The recent cessation of production of commercial indirect hemagglutination and direct agglutination kits makes another option for veterinary diagnostic testing critical. The substitution of protein A/G conjugate for the goat anti-human IgG conjugate included in the ELISA kit allows for testing across the range of animal species commonly tested. Testing on this platform does not require the extended incubation periods of agglutination testing, nor the requirement for technicians to create a serial dilution of samples, as it is a single well test. It is also a less subjective test, as optical density measurements are performed with a plate reader, rather than the tester interpreting the presence or absence and extent of agglutination visually. Finally, the ready availability of domestically produced kits and protein conjugate avoids the prolonged importation process of previously used agglutination kits and components.

Comparative testing utilized an Indirect Hemagglutination Assay (IHA) to initially test serum samples that had been submitted to the New York State Animal Health Diagnostic Center to detect anti-*Toxoplasma* IgG antibody. Another aliquot of these samples was then retested using the ELISA technique with protein A, G or A/G horseradish peroxidase as the conjugate, depending upon the animal species from

which the serum was collected and which phase of the study was being conducted. A comparison of IHA to ELISA testing using protein A for dog and cat serum and protein G for goat, horse, alpaca and sheep serum produced a Kappa value of 0.83 indicating very good agreement between the two tests, with a relative sensitivity of 90% and specificity of 95% for this group of samples. A comparison of IHA to ELISA using chimeric protein A/G conjugate for all species tested produced a Kappa value of 0.81, with a relative sensitivity of 92% and specificity of 89%. Disparate results between the two tests may be related to the underlying mechanism of detection, with ELISA requiring antibody to attach to antigen coated wells, whereas in indirect hemagglutination an antibody must be present in sufficient quantity to attach to antigen coated red blood cells to prevent agglutination. Automation of testing and the use of avidity determination of infection chronicity are additional methods that make ELISA a more attractive technology for veterinary testing, methods that have been widely employed in medical testing.

This newly developed ELISA allowed the rapid evaluation of a large number of serum samples from hunter-killed white-tailed deer across the state of New York in 2010. The prevalence in these deer was found to be 38.5%, with a 95% confidence interval of 31.2-45.8%, after correction for the ELISA test's performance sensitivity and specificity. Older deer (>1 year old) were found to have a significantly higher level of seroconversion than younger deer, providing insight into the greater significance of horizontal than vertical transmission in this population. No significant difference was noted in the level of male versus female exposure. Similarly, no significant correlation was detected between the local human population density and the risk of seropositivity

in the hunter-killed deer. Revisiting this question by including samples from deer killed in more densely populated areas of the state, for instance the Hudson Valley, will allow the opportunity to reevaluate this relationship. Other studies noted an inverse relationship between human population density and deer infection, which may be due to an increased level of infection in rural cats, or a rural cat lifestyle that perpetuates the spread of oocysts more effectively. Deer are a good sentinel for both *Toxoplasma* risk to other grazing animals of more economic significance (e.g. goats and sheep) and for the occurrence of oocysts in the local environment that also poses a risk to public health.

Detection of *Toxoplasma* DNA in tissue samples by polymerase chain reaction is commonly performed on amniotic fluid from pregnant women suspected of infection and is increasingly available in veterinary diagnostic laboratories. The most common targets are the *B1* gene and the 529 base pair repeat element. These were compared by traditional PCR with both published and newly developed primers to investigate their utility for quantitative real-time PCR detection. Primers targeting the B1 gene were incorporated into a real-time assay, with cloned target used to investigate the detection limit of the assay and the reliability of quantitation. Preparations of 40 or more target repeats produced accurate results, while those with four target repeats were detected as positive, but quantitation lacked precision. This demonstrates the sensitivity of the assay, with cycle threshold values indicating 40 or more targets being useful for quantitation. The question of the usefulness of quantitation of *Toxoplasma* DNA in veterinary medicine remains open, though the demonstration of its significance in distinguishing severe from mild infection in humans is promising. The type of tissue

sampled for PCR is critical in interpreting any results, as well as affecting the sensitivity of the assay. This was demonstrated by the lack of detection of DNA in serum samples from seropositive dogs and sheep in our study, suggesting serum as an inappropriate sample for testing. Testing blood samples from known acutely infected animals has a greater chance of detection, given that this is when tachyzoites are expected to be present in the vascular system.

Toxoplasmosis remains a disease that causes consternation among both clinicians and diagnosticians. The former due to its ability to produce a variety of clinical signs in a range of animal species, leading to its inclusion on numerous differential diagnoses lists, and the latter due to the non-specific nature of most serological test results and difficulty of acquiring appropriate tissue for molecular investigation. The development of the above ELISA technique allows the continuation of serological testing for *Toxoplasma* exposure in the veterinary diagnostic setting, which has been compromised by the termination of production of some agglutination test kits. Further development of this test to allow automation of the procedure and avidity detection will allow more efficient and informative results to be produced from a single sample. The demonstration of accurate quantitation of copy numbers in a real-time PCR assay for the detection of the *B1* gene target allows study to determine the significance in various tissue types to severity of the infection. Acquiring the appropriate tissue to accomplish sensitive antemortem detection can be challenging, though whole blood, cerebrospinal fluid, muscle punch biopsies and aborted fetal tissue are accessible and appropriate depending on the stage and localization of infection. The serosurvey of white-tailed deer from New York State demonstrates both the usefulness of the above described

ELISA technique and the potential of using deer studies to detect *Toxoplasma* in the environment. This test and sentinel species both hold promise in advancing the understanding of the risks of this common parasite to animal and human health.